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BLOCKADE OF HISTAMINE-INDUCED CONTRACTIONS OF INTESTINAL SMOOTH MUSCLE BY β-HALOALKYLAMINES

by

©

TERRENCE PETER KENAKIN

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled BLOCKADE OF HISTAMINE-INDUCED CONTRACTIONS OF INTESTINAL SMOOTH MUSCLE BY β-HALOALKYLAMINES submitted by TERRENCE PETER KENAKIN in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

In the histamine H_1 receptor system of guinea pig ileum β-haloalkylamines produce an irreversible parallel shift in dose-response curve to histamine, and, at higher doses, produce a depression of the maximum response. In order to investigate this phenomenon further, the interaction of the β-haloalkylamine phenoxybenzamine (POB), N-ethyl-N-2 chloroethyl-benzylamine (ECB), N-ethyl-N-2 chloroethyl α-naphthylamine (SY14), and N,N-dimethyl-2-bromo-phenylethylamine (DMPEA), with this receptor system were studied.

The parallel shift of dose-response curve produced by phenoxybenzamine was not abolished by the presence of thiosulphate and is thus apparently not a competitive reversible phenomenon. The insensitivity of the magnitude of the parallel shift to changes in exposure time, and the similarity of results of isotonic and isometric recordings suggests that the parallel shift in dose-response curve does not result from the presence of "spare cells".

In the absence of sodium thiosulphate, a scavenger of the aziridinium ion believed to be the active species of β-haloalkylamine, phenoxybenzamine produces a parallel shift in dose-response curve with retention of the maximum response of 0.67 log units. In the presence of sodium thiosulphate a much larger shift of about 2 log units can be achieved. The magnitude of the shift of the dose-response curve with retention of maximum response, differs significantly when ECB, phenoxybenzamine or SY14 are used.

When higher doses of phenoxybenzamine are used such that the maximum response is depressed, a small reversal of this depression is observed with prolonged washing of the tissues. This reversal is greatly accelerated by the presence of $S_2O_3^=$ and BSA in the wash fluid. The parallel shift of dose-response curve is unaffected by this treatment.

Protection of this depression of the maximum response is achieved by pretreatment with DMPEA, or receptor protection by histamine. Neither of these treatments altered the position of a dose-response curve shifted with a lower dose of phenoxybenzamine.

It was found that desensitization to histamine was capable of producing a selective protection against the phenoxybenzamine-induced depression of the maximum response. Desensitization had little effect on the shift of the dose-response curve. The results imply that desensitization for histamine involves a conformational change in the receptor.

These results suggest that there are separate processes involved in the production of the irreversible shift of the dose-response curve and the depression of the maximum response. The results do not support the "spare receptor" hypothesis but can be explained by a "two-site" hypothesis much like that proposed by Moran and Triggle (1970) for the muscarinic receptor of rat jejunum or the "two-state" hypothesis based on a model for allosteric proteins by Monod, Wyman and Changeux (1965). Calculations, based on the equation describing this latter model, predicting the effects of irreversible inhibitors of receptors are also presented.

Parts of this work have been presented earlier as follows:

T.P. Kenakin, and D.A. Cook. Antagonism of the histamine response of guinea pig ileum by beta-haloalkylamines. Presented to Canadian Federation of Biological Societies, July 1974, Hamilton, Ontario.

T.P. Kenakin, and D.A. Cook. Beta-haloalkylamine blockade of and effect of temperature on the histamine response in guinea pig ileum. Presented to Canadian Biophysical Society, November 1974, Banff, Alberta.

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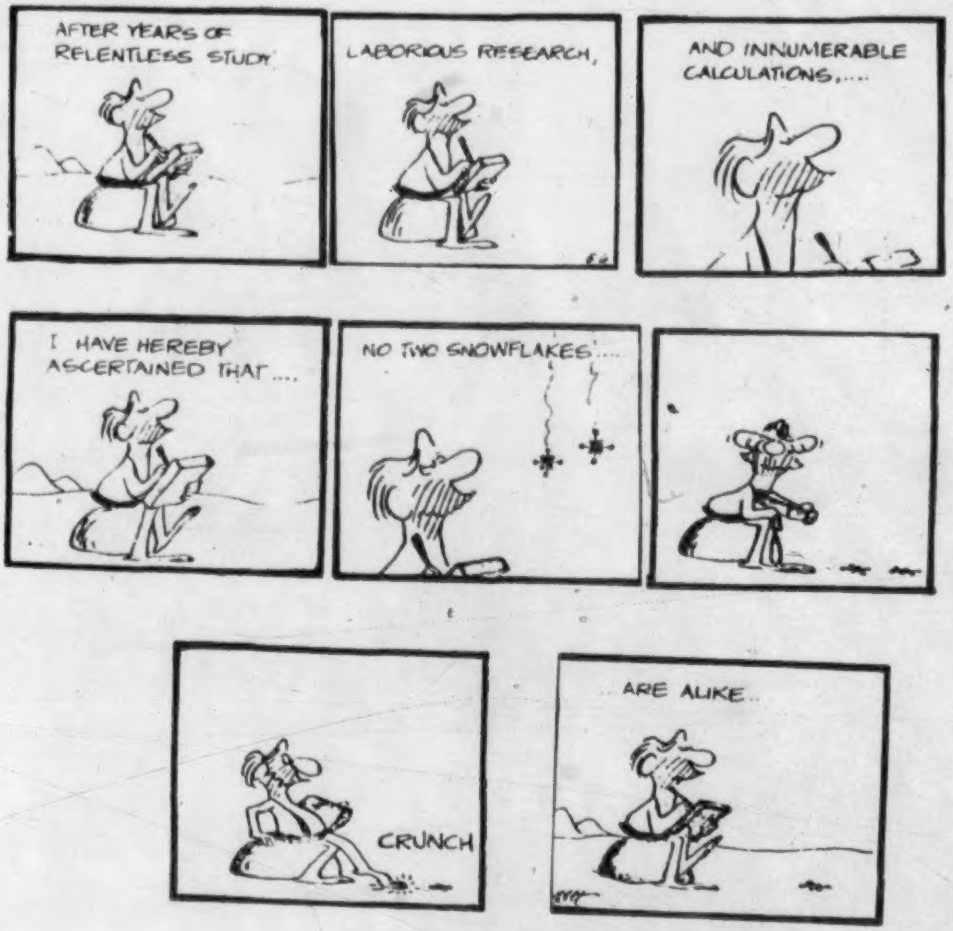
And the end of our exploring
Will be to arrive where we started
And know the place for the first time...

T. S. Eliot


AFTER YEARS OF RELENTLESS STUDY
LABORIOUS RESEARCH,
AND INNUMERABLE CALCULATIONS,....
I HAVE HEREBY ASCERTAINED THAT....
NO TWO SNOWFLAKES....
CRUNCH
...ARE ALIKE...

I. INTRODUCTION

A. GENERAL INTRODUCTION AND HISTORY

Sir William Osler (1849-1919) once said that a prime distinction between man and other creatures is man's yearning to take medicine. Whether or not this be so, most of man's knowledge about the medicines that he takes has been gained in the past 100 years. The idea that chemical substances (drugs) act on specific sites on the cell membrane (receptors) may now appear straightforward but it took many years of empirical observation before any of the processes involved in drug-receptor interaction were elucidated. The first step, was to associate disease with a biochemical etiology and not the displeasure of the Gods.

For centuries, diseases have been considered to be the result of malevolent spirits or imbalances in various humors of the body. The Ebers papyrus (circa 1550 B.C.) considered to be one of the oldest known pharmacological records offers various prescriptions to "... empty the belly and make all evil that is in the body of man come out" (Ebers, 1875). Occasionally, however, effective cures developed with no pharmacological rationale. An example of this would be the ancient Egyptian cure for night blindness: "another for night-blindness in the eyes: liver of ox, roasted and crushed out, is given against it. Really excellent." It is now known that night-blindness often results from lack of vitamin A of which liver is a prime source. A vast majority of the cures for diseases, however, were ineffective. These superstitions and metaphysical concepts in medicine persisted through to the Middle Ages and Rennaissance in spite of writings by certain non-conformists who attempted to study the etiology of disease and develop cures on a rational basis. One of these, Theophrastus Paracelsus (1493-1541) proposed that specific diseases should be treated with specific chemical substances but his words were to go unnoticed until the time of Ehrlich. With the fruitful studies of fundamental cellular processes and the success of chemists in first isolating and then synthesizing active principles from plants and animals, came the age of experimental pharmacology.

Investigators began to note that certain drugs were quite specific in their action. In 1850, Claude Bernard noted that nerves became inexcitable after treatment with curare while the muscles still reacted to direct stimulation. Inhibition of physiological processes by some chemical agents was found to be reversible and not due to unspecified toxic effects (Holmstedt and Liljestrand, 1963). In 1869, Schmiedberg and Koppe wrote: ".... muscarine does not destroy the contractile force of the heart, but only oppresses it and prevents its natural manifestations from becoming apparent...." The concept that drugs act on specific receptors began to take form.

Although Paracelsus hypothesized as early as 1500 that a drug possessed a "spicula" for binding to a tissue, credit is given to Langley (1905) for being the first to refer to a "receptive substance" as the site of action for such drugs as nicotine and curare. His experiments were essentially an extension of the work on immunity carried out by Paul Ehrlich who was impressed by his observations of high antibody specificity. Ehrlich did much to formulate the theory of receptors and wrote in 1909: "That combining group of the protoplasmic molecule to which the introduced group is anchored will hereafter be termed receptor".

There is much circumstantial evidence available on which to base the premise that there is a specific reaction between a drug and a receptor. The potency and selectivity of drugs greatly impressed A.J. Clark (1937) when he formulated the first theory describing drug action at the receptor: "The most interesting feature of drug action is the extraordinary specificity of the action of drugs and the manner in which slight changes in chemical constitution alter their action." Further substantiation of this concept is provided by the fact that some drugs can be selectively antagonized by others.

Much effort has gone into attempts to describe the action of drugs at the receptor level. Until the advent of the concept of allosterism, a drug was thought to fit directly into a receptor like a key in a lock. This idea was based on the work and writings of the great German chemist Emil Fischer who developed this theory in 1894: "... I will say that enzyme and glucoside must fit together like lock and key in order to be able to exercise a chemical action upon each other."

In 1913, Paul Ehrlich wrote: "Substances can only be anchored at any particular part of the organism if they fit into the molecule of the recipient complex like a piece of mosaic finds its' place in a pattern." This rigid "lock and key" view of drug-receptor interaction has since been recognized as being far too simple an interpretation of the physiological process. It is interesting to note that as early as 1937, Clark hinted at the theory of allosterism when he wrote: "... It is necessary to postulate a complex receptor with which one drug can unite without displacing the other drug. The antagonist drug must be assumed to alter the receptor configuration..." Although the concept is now approximately 70 years old, our knowledge of drug receptors and their mechanism of action is still fragmentary and incomplete.

The drug receptor is postulated to be a macromolecule possessing strict structural requirements for neurotransmitters, hormones and other specified chemical substances the binding of which elicit a defined response. This thesis is primarily concerned with the mechanism whereby the irreversible binding of one drug (a β-haloalkylamine) can affect the binding and subsequent production of response by another drug (histamine). Although drug receptors have often been compared to enzymes, experiments described here suffer from the fact that they must necessarily concern the receptor in situ. Thus, while the study of substrate-binding and inhibition can be carried out on purified samples of enzyme with a host of techniques, isolated tissues studies must derive all information about the drug-receptor binding kinetics from the gross phenomenon of tissue contraction. Such procedures necessitate the carrying out of experiments by the null method. Comparisons are between tissue responses obtained before and after treatment with β-haloalkylamine and the assumption is made that the observed effects are a result of changes at the drug-receptor level. Control experiments are carried out to ensure this supposition is correct, and thus there is good reason to believe that it is rational to discuss these phenomena in terms of the receptor macromolecule rather than as generalised or non-specific effects.

B. THE HISTAMINE RECEPTOR

There is overwhelming evidence that biological tissues possess receptors specific for histamine (Fig. 1). The selective antagonism of the histamine response by antihistaminic compounds and the use of pAx values (Schild, 1947) to quantitate this antagonism has demonstrated this very well. The fact that preparations may also be selectively desensitized to histamine implies that specific receptors exist for this agent (Innes, 1962; Dean, 1968). Furchgott (1954) reduced the sensitivity of rabbit aorta to many agonists with the β-haloalkylamine dibenamine (Fig. 2), and found that the sensitivity to histamine after blockade differed from that of other agonists. As dibenamine appears to act at the drug-receptor level, this strongly suggests that specific histamine receptors exist in this preparation as indicated by the differential reactivities of the various receptors to the alkylating agent. Furchgott also found that histamine could "protect" these receptors while little "cross protection" with other agonists occurred, further implying a specific histamine binding site. The various problems associated with "receptor protection", however, are a major drawback in this type of experiment and will be dealt with more fully in a later section (Waud, 1962).

Histamine causes contraction in various preparations of smooth muscle from intestine and bronchi which can be antagonized by low concentrations of antihistamines such as mepyramine (Ash and Schild, 1966). Histamine also stimulates acid secretion in the stomach (Loew and Chickering, 1941), increases mammalian heart rate (Trendelenburg, 1960) and inhibits contractions of the rat uterus (Dews and Graham, 1946). These actions, however, cannot be antagonized by low concentrations of the classical antihistamines (Trendelenburg, 1960; Ash and Schild, 1966; Douglas, 1970). Phenylethylamine has been shown to be five to ten times more active than either 2 or 4-(2-aminoethyl)-pyridine on acid secretion and rat uterus, but far less active than either of the pyridine derivatives on guinea pig ileum (Ash and Schild, 1966). This obvious dichotomy in the action of histaminergic agents led Ash and Schild (1966) to propose the division of the known population of histamine receptors into two types: H_1 for the receptors on bronchi and gut and H_2 for the receptors on rat uterus, the heart, and the stomach. In certain preparations of vascular smooth muscle, both H_1 and H_2 receptors have been characterized (Edvinsson and Owman, 1974; Grennan et al., 1974).

Work began in 1964 at the research laboratories of Smith, Kline and French toward the synthesis of a specific H_2 receptor antagonist and after the testing of seven hundred compounds, burimamide was discovered. This agent was found to be a sufficiently selective surmountable antagonist of histamine on atria and satisfied all criteria for competitive reversible receptor blockade (Black et al., 1972). The pA_2 for atria corresponded to that for rat uterus while histamine effects on cat blood pressure, normally refractory to mepyramine (Folkow et al., 1948), were selectively antagonized by burimamide. It was also found that burimamide antagonized gastric secretion in animals (Black et al., 1972), in man (Wyllie et al., 1972) and in isolated preparations (Black et al., 1972). This group of investigators has since produced metiamide, another H_2 antagonist with low toxicity (Black et al., 1973). With the coming of this new group of selective H_2 receptor antagonists, little doubt remains about the validity of the classification of histamine receptors into these two subgroups.

The cardiac H_2 receptor has been associated with increases in phosphorylase "a" levels in cells (Poch and Kukovetz, 1967) and increases in cellular cyclic AMP levels (McNeill and Verma, 1974). Although there is suggestive evidence at present, no definite relationship between the H_2 receptor and the adenyl cyclase system has been established. Recent studies suggest that H_1 and H_2 receptors are forms of the same macromolecule but the evidence is as yet inconclusive (Kenakin, Krueger and Cook, 1974; Okpawa, 1975; Cook, Kenakin and Krueger, 1975). As the experimental work discussed in this thesis deals with the alkylation of the histamine receptor in longitudinal smooth muscle from guinea pig ileum, all further references to histamine receptors will refer to the H_1 receptor unless otherwise specified.

There is substantial evidence that the H_1 receptor is one of a family of "integral membrane proteins" (Singer, 1971; Singer and Nicholson, 1972) and that the active site is exposed to the extracellular space. Histamine is a strong base with a pKa of 9.7 for the side chain amino group and 5.8 for the imidazole nitrogen and therefore exists almost exclusively as a univalent cation at physiological pH (Levy, 1935; Holmes and Jones, 1960). Histamine effects on smooth muscle occur very quickly and an intracellular receptor dependent on a fast transport system for the univalent cation is unlikely. The responses to histamine are pH dependent implying that the active site is under the influence of the pH in the extracellular fluid (Rocha e Silva, 1966). The alkylating agents, β-haloalkylamines, known to antagonize drug responses through a positively changed cyclic aziridinium ion, (see later section) block the histamine response and although evidence exists that these agents can enter the cell (see later section), the characteristics of the blockade strongly suggest that the alkylation takes place at the extracellular surface of the membrane. Quaternization of certain antihistaminic compounds does not abolish their activity with respect to the histamine receptor further implying an extracellular locus (Ariëns, 1967).

Generally speaking, the structural requirements for agonist properties at the H_2 receptor can be summarized as follows: i) a basic side chain appears to be essential for activity ii) chains shorter or longer than the ethyl group render the molecule inactive while substituents on the terminal amino group reduce activity iii) the position of attachment of the side chain on the imidazole ring is critical iv) addition of a methyl group to the imidazole ring reduces activity v) aminoethyltriazole, pyrazole, thiazole and pyridine analogs are less active (Jones, 1966; Paton, 1973). Jones (1966) concluded, after an exhaustive study on a series of analogs that "... compounds possessing appreciable histamine-like activity consist of small nitrogen heterocyclic aromatic rings to which are attached 2-aminoethyl side chains".

Much work has been done to determine the nature of drug receptors and in general, treatments and procedures known to affect protein (enzymes, lipid solvents, sulphydryl reagents, protein denaturants, chelating agents, heat and cold) also alter drug effects at the receptor level (Ehrenpreis et al., 1969). Although most of that work concerns the acetylcholine receptor, certain specific studies with histamine imply a protein nature for this receptor as well. A potent antihistaminic β-haloalkylamine N-ethyl-N-(β-bromoethyl)-1'-naphthylmethylamine (SY28) (Fig. 3) has been shown to alkylate various amino acids of protein in the guinea pig vas deferens, the residues being histidine, arginine, aspartic acid, serine and methionine (Graham and Mottram, 1971; Mottram, 1974). However, this is tenuous evidence when viewed in light of the known non-specificity of these alkylating agents (vide infra, Section E). A study of pH and responses to histamine has shown that the active site of the histamine receptor appears to contain an ionizable group with pKa of 7.0 (Rocha e Silva, 1960). A study by Ariëns and Simonis (1965) has led to the conclusion that there is a group of pKa 6.8 in the histamine receptor. The imidazole ring is the only group known to have a pKa in this region. Thus it has been suggested that the active site of the H_1 receptor contains a histidine residue (Rocha e Silva, 1960, 1969a).

The degradative enzyme diamine oxidase (histaminase) has been detected in preparations of trachea, ileum and uterus. A study by Arunlakshana, Mongar and Schild (1954) has shown that antagonism of this enzyme has effects on the dose response curve to histamine (the tissue appears to be more sensitive) but careful controls can account for any effects of histaminase. Certain β-haloalkylamines, however, are known to block histaminase (James, 1959) and the enzyme must be considered in receptor protection studies.

C. β-HALOALKYLAMINES - A SHORT HISTORY AND GENERAL EFFECTS

The prototype β-haloalkylamine, dibenamine, was first described chemically by Eisleb (1934) and prepared in North America by William Gump. This compound closely resembles the nitrogen mustard gases used in World War I but lacks the second chloroethyl group. Eleven years after the synthesis of dibenamine came a report of this agent's remarkable ability to reverse the pressor response of adrenaline injected into a cat (Nickerson and Goodman, 1945). This publication was rapidly followed by a series of reports describing the pharmacology of this compound (Nickerson and Goodman, 1946, 1947). In their classic paper, Nickerson and Goodman (1947) showed that it was impossible to overcome the blockade with injected adrenaline and that the antagonism was present for three to four days. So much had dibenamine captured the interest of pharmacologists, that in a review published two years later, Nickerson (1949) was able to cite over one hundred references to the drug. Dibenamine has since been superceded by more active β-haloalkylamines with more reliable blocking properties (Graham, 1962) such as phenoxybenzamine (Fig. 4). Most of the research done on β-haloalkylamines concerns their antiadrenergic activity. As well as the well documented effects on the pressor response, these agents are known to be irreversible antagonists of catecholamines on rabbit aorta (Furchgott, 1954), isolated seminal vesicles of the guinea pig or rat (Leitch _et al_., 1954), uterus of the rabbit or pregnant cat, perfused vessels of the rabbit ear, and vessels of the hind quarters of small animals (Graham, 1962). Reports have indicated that β-haloalkylamines block the action of circulating catecholamines much more easily than they block the effects of adrenergic nerve stimulation (Nickerson, 1959), but there is conflicting evidence in this regard (Wyse and Beck, 1972). β-Haloalkylamines do not block the catecholamine induced stimulation of mammalian cardiac muscle (Nickerson and Chen, 1961) but do antagonize catecholamine effects on the heart of the frog and other amphibia (Graham, 1959). These agents do not block the relaxation of smooth

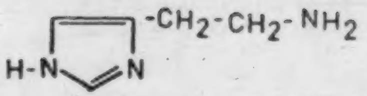

-CH2-CH2-NH2
H-N
N

Figure 1. Histamine

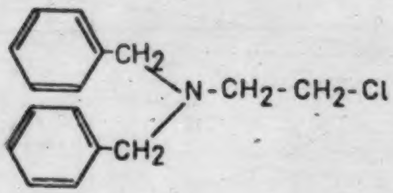

CH2
N-CH2-CH2-Cl
CH2

Figure 2. Dibenamine

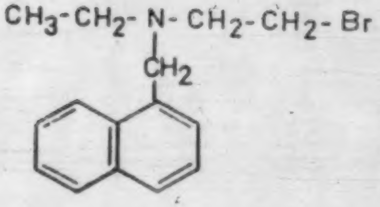

CH3-CH2-N-CH2-CH2-Br
CH2

Figure 3. N-ethyl-N-β-bromoethyl)-1'-naphylmethylamine (SY28). The structure of SY14 is identical but for the substitution of chlorine for the bromine.

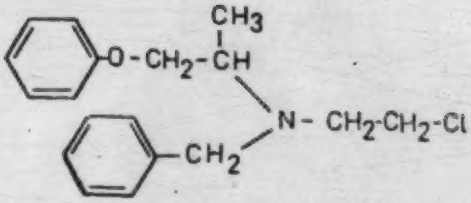

CH3
O-CH2-CH
N- CH2-CH2-Cl
CH2

Figure 4. Phenoxybenzamine

muscle to catecholamines (Agarwal and Harvey, 1955) but very high concentrations can partially antagonize the adrenaline specific dilatation of vascular smooth muscle (Youmans, Green and Denison, 1955). The action of these agents on the adrenergic system have now been fairly well characterized. They block the α-receptor directly (Nickerson and Goodman, 1947), block neuronal catecholamine uptake sites (Iversen, 1965), block presynaptic sites which normally exert a "negative feedback" on the release of noradrenaline by adrenergic nerves, block the storage of noradrenaline at nerve granules (von Euler, 1964) and inhibit extraneuronal transport sites leading to a decrease in the metabolism of noradrenaline (Eisenfeld et al., 1967). Although it has been stated that certain β-haloalkylamines do not antagonize cholinergic receptors (Graham, 1952), it is now known that these agents block the actions of a variety of agonists in many preparations (Furchgott, 1954; Cook, 1971). They are non-specific alkylating agents binding to many sites and interfering with many processes (Yong and Marks, 1967; Terner, Cook and Marks, 1971).

Many β-haloalkylamines have a profound local anesthetic action. The agent N,N-bis-2-phenoxyethyl-2-bromoethylamine, for example, is a much better local anesthetic than procaine (Graham, 1960). Some of these agents, in aqueous solutions, hydrolyze to form alcohols which act as potent local anesthetics (Furchgott, 1954; James, 1959) and these actions must often be differentiated from the effects of receptor blockade by the parent β-haloalkylamine.

β-Haloalkylamines have been used to elucidate the active site of enzymes (Crestfield et al., 1963; Oosterbaan and Cohen, 1964; Harris, 1964) and some have considered the logical extension of such work to be the specific labelling of membrane receptors (Takagi et al., 1965; Moran et al., 1967; Takagi and Takahashi, 1968). The procedure in these studies is to specifically label the receptor in situ with radioactive β-haloalkylamine, homogenize and fractionate the various subcellular species, and isolate the entity bound to the radioactive drug (Lewis and Miller, 1966; Yong et al., 1966; May et al., 1967; Yong and Marks, 1969; Moran and Triggle, 1970). The conclusion was quickly reached, however, that these agents do not possess the specificity required for such a procedure. It must be noted, as well, that the activity of the receptor component may depend on the integrity of the cell membrane (Trams, 1964) and thus activity would be lost in the fractionation and isolation procedure. The behavior of the isolated receptor protein may well be anamolous and different from that in the membrane or in the words of A.K. Balls (1958). "A relationship may well exist between protein and Proteus - a sea god who changes form whenever submitted to "scrutiny". Conclusive evidence that the isolated protein represents the receptor is difficult to obtain since there would be no contractile machinery available to give a specific response.

β-Haloalkylamines have also been used to determine ligand affinity constants for receptors (Ariëns, 1964; Furchgott, 1966; Furchgott and Bursztyn, 1967; Waud, 1968a, 1968b) but recent studies imply that such data could be totally erroneous (Moran and Triggle, 1970). Receptor-occupancy relationships have also been derived from studies utilizing β-haloalkylamines (Ariëns, 1964; Furchgott, 1966; Furchgott and Bursztyn, 1967; Waud, 1968a, 1968b; May et al., 1967; Moran and Triggle, 1970) but recent reports indicate that such data may be artifacts as well (Burgen and Spero, 1968; Moran, Triggle and Triggle, 1969).

As there is some evidence that these agents interact directly with the receptor and since the blockade is long lasting, the term competitive irreversible has been adopted to describe the receptor antagonism (Kimelberg, Moran and Triggle, 1965).

Although the receptor effects of these agents most likely takes place on the extracellular surface of the membrane, recent evidence shows that agents such as phenoxybenzamine are capable of entering into the cell cytoplasm as well as parts of the cell nucleus (Graham *et al*., 1968; Graham *et al*., 1971; Mottram, 1974).

D. CHEMISTRY OF THE β-HALOALKYLAMINES

There is a great deal of evidence available to implicate the aziridinium ion as the active antagonist of the drug receptor (Fig. 5). Relatively good correlations between the amount of aziridinium ion formed by various compounds and their adrenolytic and antihistaminic activity have been obtained (Graham and Lewis, 1954; Graham, 1957). All active β-haloalkylamines studied form the aziridinium ion (Nickerson and Goodman, 1947; Belleau, 1958) and some aziridinium ion-picrylsulphonate complexes have been isolated (Graham, 1957). Those picrylsulphonates with sufficient solubility have been shown to possess antiadrenergic and antihistaminic properties (Allen and Chapman, 1960; Graham, 1960).

The adrenolytic potency correlates well with the ease with which the halogen leaves during formation of the aziridinium ion, the order being $\mathrm{I} \sim \mathrm{Br} > \mathrm{Cl} > \mathrm{F}$ (Hunt, 1949; Nickerson and Gump, 1949; Graham and Lewis, 1953; Graham, 1959). The fluoro compounds do not form the aziridinium ion (Graham, 1957) and, as expected, possess no

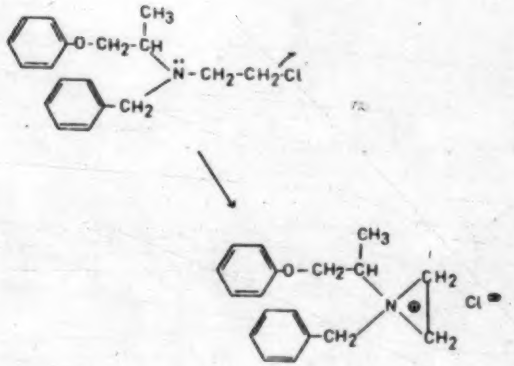


Figure 5. Formation of aziridinium ion for phenoxybenzamine

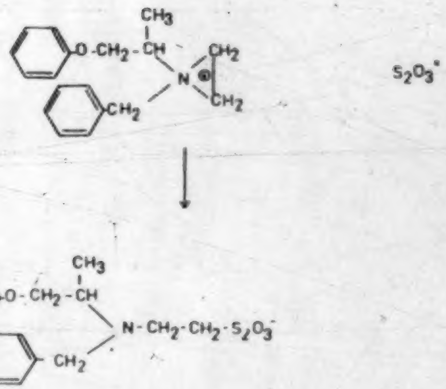


Figure 6. Formation of the Bünte salt from phenoxybenzamine and sodium thiosulphate aziridinium ion

antagonistic activity (Chapman et al., 1951). Aziridinium ions form inactive Bünte salts (Fig. 6) with thiosulphate ion (Bünte, 1874; Fruton, Stein and Bergman, 1946) and it is well known that the antagonism is diminished or even abolished by thiosulphate ion (Nickerson and Goodman, 1947; Graham, 1960b). Rosen and coworkers (1973) have suggested that the stability of the aziridinium ion ring is of the utmost importance in determining whether the molecule will alkylate or exist as the ion in solution. It is interesting to note that some of the compounds tested had very stable aziridinium ion ring systems and could exist in solution for many hours. Further interpretation of their findings is difficult, however, as details of conditions were not given. In a study on the mechanism of alkylation by these compounds, Belleau and Triggle (1962) have shown that an sn_1 type of mechanism is operative with significant shielding of the formed carbonium ion. When primarily d or l β-haloalkylamine was allowed to cyclize, there was 45% racemization and 55% net retention of configuration. These investigators thus viewed the aziridinium ion ring structure as a shielded carbonium ion (Fig. 7).

Although the aziridinium ion has been shown to be the active form, it is not the only species formed in solution. Nickerson and Gump (1949), in an early study, concluded that a quaternary imino group was an important intermediate (Fig. 8) but subsequent investigation has shown this not to be the case. A vinylamine species was thought to be another possible candidate but these have also been shown to be inactive (Graham, 1960a) (Fig. 9).

A substantial quantity of piperazinium compounds (Fig. 10) form during cyclization but a study in which Nickerson and Gump (1949)

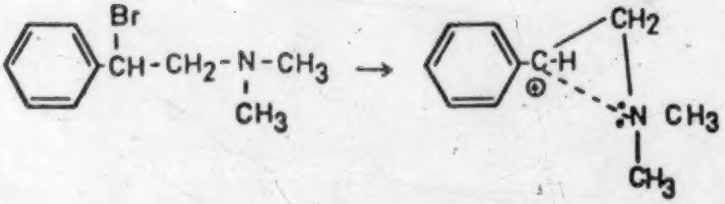


Figure 7. Aziridinium ion represented as a secondary carbonium ion shielded and stabilized by the electron pair of the nitrogen atom.

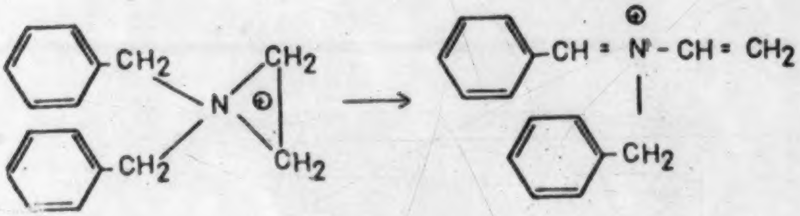
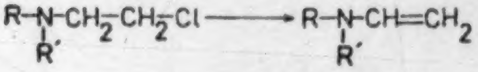


Figure 8. An early representation of an active intermediate from the aziridinium ion (Nickerson and Gump, 1949).



$$R-\underset{R'}{N}-CH_2-CH_2-Cl \longrightarrow R-\underset{R'}{N}-CH=CH_2$$

Figure 9. Formation of a vinylamine species from the aziridinium ion (Graham, 1960a).

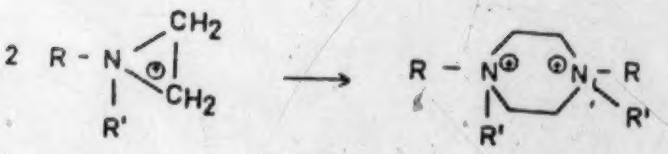


Figure 10. Formation of a piperazinium compound from 2 aziridinium ions (Nickerson and Gump, 1949).

prepared a series of these compounds eliminated the possibility that antagonism was due to these species; they proved to be uniformly inactive. Aziridinium ions in aqueous solution react with water to form alcohols and although many of these compounds can be potent local anesthetics, no alcohol derivative has, as yet, been shown to have appreciable receptor-blocking properties (Nickerson and Gump, 1949; James, 1959; Graham and James, 1961).

Structure-activity studies have elucidated a number of general rules about the relationship between adrenolytic and antihistaminic activity, and chemical structure. A chain of two carbon atoms appears to be essential in the halogen containing group (Nickerson and Gump, 1949; Graham and James, 1961) and increasing the length of the carbon chain decreases or abolishes activity. It is also essential to have at least one of the N-substituents as an aromatic ligand (Graham, 1962). A substituent larger than a methyl group on the aromatic ring greatly diminishes activity and Rocha e Silva (1960) has inferred that because these radicals lie off the plane of the ring they upset the fit of the molecule onto the receptor. If one substituent on the nitrogen atom is an aminoethyl group (Fig. 11) all activity is lost through the formation of a six membered ring (Nickerson and Gump, 1949; Ullyot and Kerwin, 1956). When the nitrogen is part of a ring system as in N-(2-chloroethyl)-piperidine (Fig. 12), the molecule is usually inactive (Nickerson and Gump, 1949).

If the compound is a quaternary salt, as in N,N,-diethyl-N-(2-chloroethyl)-carbazole (Fig. 13), it is inactive as a blocking agent (Ullyot and Kerwin, 1956).

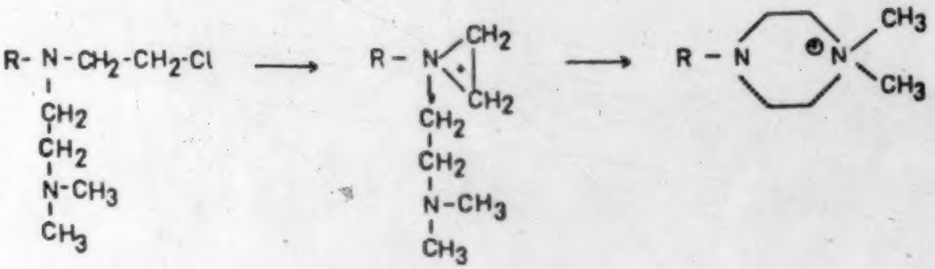


Figure 11. Mechanism for the loss of activity observed with β-haloalyllamines possessing an aminoethyl group on the nitrogen atom (Nickerson and Gump, 1949; Ullyot and Kerwin, 1956).

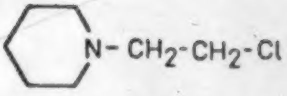


Figure 12.

N-(2-chloroethyl)-piperidine

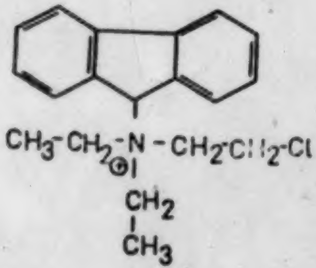


Figure 13.

N,N-diethyl-N-(2-chloroethyl)-carbazole

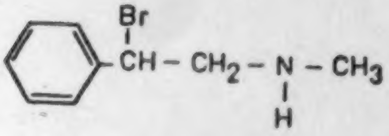


Figure 14 N-methyl-1-bromophenylethylamine

Except for a series of compounds in which a secondary amine provides an active antagonist (N-methyl-1-bromoethylphenylamine) (Graham, 1962) (Fig. 14), a tertiary amino group is in general essential (Nickerson, 1949).

E. MECHANISM OF ACTION OF β-HALOALKYLAMINES

Initial studies with dibenamine suggested that, by analogy with reactions of nitrogen mustards, the β-haloalkylamines form a covalent bond with the receptor (Nickerson and Goodman, 1947; Nickerson, 1962). This is probable in light of the fact that thiosulphate wash for periods of up to twelve hours results in no reduction of blockade (Graham and Lewis, 1954). It was soon recognized, however, that β-haloalkylamines have two stages of antagonism; an initial reversible phase followed by an irreversible stage (Nickerson and Harris, 1949; Nickerson and Gump, 1949; Nickerson, 1956). Thus it appears that the aziridinium ion binds to the receptor in a competitive reversible manner and subsequently alkylates.

There have been many studies directed at elucidating the site of action for these drugs. On a gross scale, it was noted that ^{14}C-phenoxybenzamine, when injected into mice, tended to accumulate in soft tissues such as liver and kidneys, and remained there for up to twenty-four hours (Nikawitz et al., 1952). The fact that β-haloalkylamines are quite non-specific and bind to many sites on the cell membrane (Yong and Marks, 1967; May et al., 1967; Cook, 1971) is in keeping with the hypothesis that these agents alkylate protein. Mottram (1970) was able to recover bound radioactive SY28 with papain and concluded that this agent binds to protein to cause the observed antagonism of response. As stated earlier, these agents irreversibly bind to enzymes and further studies have shown that they can alkylate components of protein. Fruton and coworkers (1946) demonstrated that mustard gases alkylate many amino acids in vitro at various electrophilic sites such as the ε -amino group of lysine, the imidazole of histidine, the sulphide group of methionine, the β or γ carboxyl group of aspartic and glutamic acid, various sulphydryl groups and any free amino or carboxyl groups on the end of peptide chains. Phenoxybenzamine is known to alkylate the sulphydryl group in dimercaprol and cysteine, inorganic phosphate and many amino and carboxyl groups of peptides (Graham, 1960). Graham (1960) used cadmium chloride to chelate phosphate and found that this reduced the reactivity to β-haloalkylamines thereby further implicating this group as a possible binding site. Belleau (1958, 1960) also presented evidence that these agents bind to phosphate and carboxylate groups.

On the basis of a study in which trypsin was utilized to affect recovery from blockade by SY28, Graham and Al Katib (1966) proposed that this agent binds to a free carboxyl group at the adrenergic receptor. Care must be taken, however, in interpreting these results since Moran and coworkers (1967) were able to achieve the same recovery with subtilisin, phosphodiesterase, and trypsin. They concluded that since the three widely different treatments could elicit recovery, a nonspecific effect related to tissue damage was probable.

Dikstein and Sulman (1965) have proposed the β-haloalkylamine binding site is a lipid. They found that all of the radioactivity associated with ^{14}C could be extracted with organic solvents. Yong and coworkers (1966, 1967) were unable to reproduce their findings and in light of the large body of evidence to the contrary, it is unlikely that the primary site of action, for these drugs, is lipid.

Recently it has been suggested that β-haloalkylamines antagonize the wide variety of agonists in smooth muscle by blocking a site common to all of them (Moran et al., 1970; Swamy and Triggle, 1972). Some evidence suggests that these antagonists also block potassium contractions (Bevan et al., 1963; Shibata and Carrier, 1967; Shibata et al., 1968) but there is opposing evidence to this view (Cook, 1971). Finally, it is known that dibenamine blocks calcium-induced but not potassium-induced contractions at a concentration which blocks the noradrenaline response (Somlyo and Somlyo, 1969). These results, and others taken together, led to the hypothesis that β-haloalkylamines bind to at least two sites intimately associated with the adrenergic receptor; one being responsible for calcium mobilization in contraction (Moran et al., 1970). Theories concerning the mechanism of blockade of cholinergic and histaminergic receptors by these agents more appropriately belong in the section on spare receptors and two-site theories (*vide infra*).

Two possible mechanisms have been proposed for the slow but measurable recovery from β-haloalkylamine blockade. Studies in which trypsin has elicited recovery lend support to a theory of an enzymic mechanism of recovery (Graham and Al Katib, 1966; Graham and Mottram, 1971; Mottram, 1974). This is a possibility *in vivo* where circulating enzymes exist but does little to explain recovery in isolated tissue preparations. An alternative proposal involves an intramolecular assisted-ester hydrolysis at the receptor (Belleau, 1958). Kimelberg and Triggle (1965) were able to correlate the nucleophilicity of the amino function with the ease of cyclization and further correlated the rate of onset with the duration of blockade. Their study implied that the same chemical factors could be controlling both the rate of onset and duration of blockade. There is abundant evidence for the existence of intramolecular nucleophilic participation in the hydrolysis of carboxylic acid esters (Fig. 15) (Bender, 1960; Bruice, 1962; Capon, 1964).

This hypothesis is further supported by the fact that first order rate constants for recovery were observed which correlated with the nucleophilicity of the amino group. The advantage of this model is that no external agent, such as an enzyme, is required but it suffers from the fact that the complex must involve a carboxylic acid ester or phosphate ester bond.

Theoretically, another possible mechanism of recovery would be the synthesis of new drug receptors and destruction of blocked ones. Little data is available at present, however, on which to consider the merits of this theory.

F. ANTIHISTAMINIC PROPERTIES OF β-HALOALKYLAMINES

It was soon recognized that many β-haloalkylamines possess significant antihistaminic properties (Achenbach and Loew, 1947; Loew, 1947; Loew and Micetich, 1947; Nickerson et al., 1948) as well as adrenolytic activity. Although it was noted that dibenamine was a rather poor antihistamine, phenoxybenzamine was considerably more active in this regard (Nickerson and Harris, 1949). These workers were able to protect against phenoxybenzamine blockade with the antihistamine diphenhydramine (Fig. 16) and thus concluded that the irreversible agent

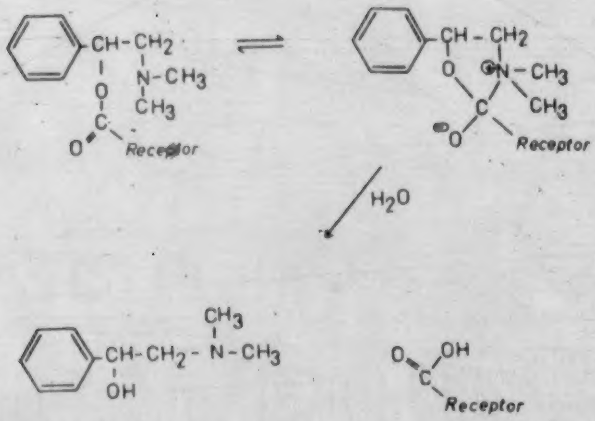


Figure 15. Assisted ester-hydrolysis. Mechanism for intramolecular nucleophilic participation in the hydrolysis of an ester bond formed between a β-haloalkylamine and a carboxyl group (being part of the receptor).

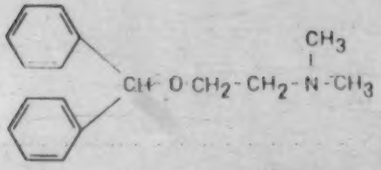


Figure 16. Diphenhydramine

reacted with histamine receptors. Naphthyl-1-methyl β-haloalkylamine derivatives, of which SY28 is the most often used, were even more potent antihistamines than phenoxybenzamine and a structure activity study showed that factors favoring adrenolytic activity in this molecule favor antihistaminic activity as well (Graham and Lewis, 1953). There were, however, vast differences in the duration of action, the antihistaminic activity lasting far longer than blockade of adrenaline. No generalizations can be made about structure activity relationships for antihistaminic activity since a later study showed that the most potent β-haloalkylamines for adrenolytic activity were not necessarily the most potent antihistamines (Graham, 1960). The classic study on histamine blockade on the H_1 receptor was carried out using the agent SY28, by Nickerson (1956). A detailed analysis of these experiments follows in a later section but generally it was found that diphenhydramine produced complete protection and histamine a partial protection against SY28 blockade. The blockade could not be reversed with up to 8 hours of wash and it was concluded that SY28 specifically alkylated H_1 receptors.

It is very probable that the aziridinium ion is responsible for the blockade of response to histamine as it is for the blockade of adrenergic receptors. There have been studies showing that this antihistaminic activity can be correlated with levels of aziridinium ion (Graham, 1957) while aziridinium ions isolated as picrylsulphonates are potent antihistamines (Graham, 1960).

G. THE SPARE RECEPTOR HYPOTHESIS

The concept that only a fraction of the existing receptors need be activated for the production of maximum response was proposed on theoretical grounds by Stephenson (1956). A large proportion of the receptors would thus be deemed "spare" and the remaining fraction required for maximum response, the "critical fraction". There have been attacks, on the theoretical level, however, on the Clark-Ariëns-Stephenson model of drug-receptor interaction on which the "spare receptor: hypothesis is based (Triggle, 1965; Belleau, 1965). The classic experiment performed by Nickerson (1956) initially appeared to provide an experimental basis for this theory. By manipulating the dose of the irreversible agent SY28, Nickerson was able to attain a 2 log unit shift in the dose-response curve to histamine, with retention of maximum response. Such a finding is usually indicative of competitive reversible antagonism, but as stated earlier, washing for up to 8 hours failed to reverse the blockade. Higher concentrations of blocking agent irreversibly depressed the maximum response. Nickerson reasoned that approximately 1 percent of the receptors were required to be activated for maximum response. Irreversible antagonism of the other 99 percent, or spare receptors, made higher concentrations of histamine necessary in order to achieve the 1 percent receptor activation for maximum response. These higher concentrations of agonist required for equivalent responses were reflected in the shift of the dose-response curve and until more than 99 percent of the receptors were blocked, the maximum response could always be achieved. With greater exposure to the irreversible antagonist this last critical percentage of the receptors is blocked and the maximum response is depressed. Since the initial observation, there have been widespread reports of receptor reserves in other preparations (Ariëns et al., 1960; Furchgott, 1966; van Rossum et al., 1966a, Furchgott and Bursztyn, 1967) but different experimental procedures have shown many of these reserves to be artifact (Ariëns, 1964; Lewis and Miller, 1966; May et al., 1967; Moran, Triggle and Triggle, 1969; Cook, 1970). The irreversible parallel shift in dose response curve found for various cholinergic preparations (Ariëns et al., 1960; van Rossum and Ariëns, 1962; Burgen, 1965; Furchgott and Bursztyn, 1967) and that found in guinea pig ileum for histamine (Nickerson, 1956) do not appear to be an artifact of experimental procedure and thus it is still widely believed that a receptor reserve exists in these preparations. Estimates of the actual percentage of spare receptors in these preparations range from 99% to 99.9999% (Nickerson, 1956; Schild, 1962; Paton and Rang, 1966).

There have been other hypothesis put forward to explain this irreversible shift of dose-response curves produced by β-haloalkylamines. Waud (1968b) has proposed that perhaps "spare cells" and not "spare receptors" are responsible for the phenomenon but opposing evidence tends to dispute this theory for histamine H_1 receptors (Cook, 1971; Kenakin and Cook, 1975). As noted earlier, β-haloalkylamines have a distinct competitive reversible phase of action before alkylation and the possibility that this shift in dose-response curve is due to reversible binding of aziridinium ion has been proposed on theoretical grounds (Triggle, 1965). In the rat vas deferens it was found that the parallel shift induced by SY28, in the dose response curve to noradrenaline, was reversed by thiosulphate ion (Moran, Triggle and Triggle, 1969). Since thiosulphate cannot affect an alkylated drug-receptor complex but can react with aziridinium ion, it was evident that what appeared to be a receptor reserve in this tissue preparation was really a competitive reversible effect of the aziridinium ion.

Another approach to this problem has been the comparison of rates of recovery from blockade for full and partial agonists. Partial agonists, by definition, must occupy the total receptor population to produce their maximum response (Stephenson, 1956; Trendelenburg, 1963; Ariëns, 1964). If a large proportion of the receptors are "spare" for the full agonist, then the rates of recovery to this full agonist and a partial agonist should differ considerably. May and coworkers (1967), found, however, the half-times for the recovery of response for full and partial adrenergic agonists in rabbit aorta, to be about equal thereby making the existence of a receptor reserve in this tissue highly unlikely.

Establishing an alternative to the spare receptor hypothesis in cholinergic systems and the H_1 receptor system in guinea pig ileum has proven to be more difficult. The parallel shift in both of these systems is insensitive to thiosulphate wash and thus seems to be due to an alkylated drug-receptor complex. Unlike the α-adrenergic receptor system, rates of recovery to full and partial agonists in the cholinergic system are not equal (Moran and Triggle, 1970), a finding which would tend to support the spare receptor hypothesis.

Certain results in the cholinergic receptor system in smooth muscle cannot fully be explained by this theory. Burgen and Spero (1968) found through studies with reversible muscarinic antagonists, that the receptors responsible for potassium efflux out of longitudinal smooth muscle were of the muscarinic type. Dibenamine, while producing a shift in the dose response curve for contraction to acetylcholine, caused an immediate depression of maximum in the dose response curve for potassium efflux. These results are not in any way amenable to explanation by the spare receptor hypothesis and at the very least, as Burgen points out, make estimations of receptor reserve with agents such as dibenamine highly suspect. Burgen and Spero (1968) go on to propose that there exist two types of muscarinic receptor in this preparation. Another hypothesis capable of providing an explanation for anamalous results on cholinergic preparations with β-haloalkylamines is conceptually similar, but involves two binding sites for muscarinic agonists instead of two distinct receptors. Consideration of this model and its ramifications more properly belong to the following section.

H. TWO-SITE THEORIES AND ALLOSTERISM IN RECEPTORS

From elegant studies on the blockade of the cholinergic response in intestinal smooth muscle by β-haloalkylamines, Moran and Triggle (1970) obtained results which do not support the spare receptor hypothesis. As stated before, partial agonists must activate one hundred percent of the receptor population in order to produce maximal responses. Thus, recovery of the maximal response to partial agonists during reversal of blockade from β-haloalkylamines (see section E) should proceed at the same rate. The maximal response to the partial agonist must depend on the number of unblocked receptors, which at any given time will be equal for all partial agonists during the washoff of β-haloalkylamine from the receptor population (or the generation of new receptors). It was found, however, that recovery of the maximum response to three partial agonists differed considerably. Moreover, the washout of radioactive β-haloalkylamine was clearly biphasic and reflected washoff of antagonist from two distinct membrane sites. The half-time ($t_{1/2}$) for removal of antagonist from one site closely corresponded to the $t_{1/2}$ for recovery to full agonists while the removal of blocker from the second site correlated with recovery of response to partial agonists. This led Moran and Triggle (1970) to formulate a "two-site" model for the muscarinic receptor. As shown in Fig. 17, a common binding site exists for the quarternary head of all agonists with two other binding sites adjacent to it. One of these adjacent sites binds acetylcholine-like compounds while the other binds the alkylammonium-like compounds which are partial agonists. Binding of the β-haloalkylamine to the alkylammonium binding site produces an allosteric perturbation of the acetylcholine binding site causing the parallel shift in dose response curve with no depression of maximum. Partial agonists, which would normally bind at this site, would produce depressed maximum responses. This would be in accordance with the finding that β-haloalkylamines produce only a depression of maximum response to partial agonists (Ariëns *et al.*, 1960). Further alkylation would involve the common anionic site and results in a depression of maximum response to acetylcholine-like agonists. Such a hypothesis is very flexible in that the chemical structure of the agonist and antagonist would determine both the extent of the shift of the dose-response curve and the $t_{1/2}$ for recovery of response. The various properties of β-haloalkylamine blockade would thus not be a function of the number of spare receptors in the tissue, but rather of the chemical properties

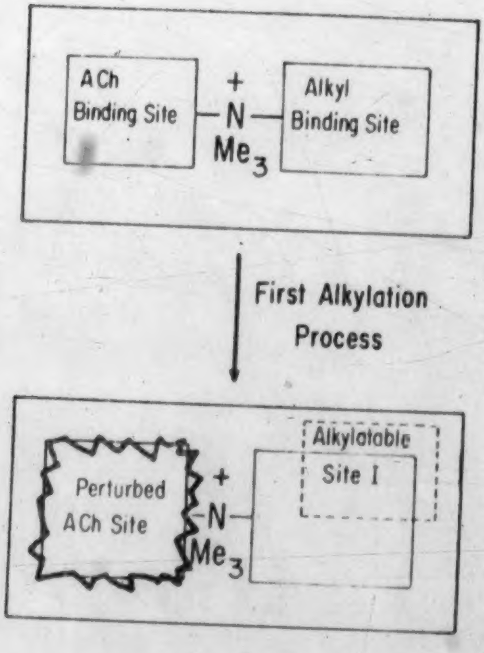

ACh
Binding Site
+
N
Me$_3$
Alkyl
Binding Site
First Alkylation
Process
Perturbed
ACh Site
+
N
Me$_3$
Alkylatable
Site I

Figure 17. Schematic view of the two-site hypothesis for the muscarinic receptor of intestinal smooth muscle (reproduced from Moran and Triggle, 1970).

of the agents used to study the antagonism. Recent studies by Ross and Triggle (1974) provide further support for this allosteric view of the muscarinic receptor. Certain aryldiazonium salts produce only a parallel shift in the dose response curve to carbachol with no accompanying depression of maximum response. Furthermore, agonists do not show preferential protection against blockade of maximum response by agents which depress the maximum response as would be expected if depression were caused by alkylation of a very small critical fraction of receptors. Protection against shift and depression occurred concurrently and with equal facility.

There is much circumstantial evidence available to show that the cholinergic receptor exhibits allosterism. Kitz and coworkers (1969) have provided evidence that acetylcholinesterase is an allosteric protein as have the NMR studies of Kato and Yong (1971). Although there is considerable controversy over whether acetylcholinesterase is a proper model for the acetylcholine receptor, it is nevertheless a protein with specific binding properties for acetylcholine. In a study concerning the stereoselectivity of various benzylic esters on the acetylcholine receptor, Ellenbroeck and coworkers (1965) and Ariëns and Simonis (1967) concluded that atropine binds to a site separate and distinct from that for acetylcholine and elicits blockade of the receptor through an allosteric mechanism. This same conclusion has been reached by other investigators as well (Goldstein et al., 1968; Geddes et al., 1974). As mentioned earlier, two-site theories are not restricted to cholinergic receptors but have a precedent in the adrenergic receptor system (Moran, Swamy and Triggle, 1970; Janis and Triggle, 1971; Swamy and Triggle, 1972; Kalsner, 1973).

A similar theory has been proposed for the histamine H_1 receptor after it was observed that recovery from blockade by the antihistamine diphenhydramine was not accelerated by very high doses of histamine as would be expected if the two agents were interacting at a common site (Beraldo and Rocha e Silva, 1949; Rocha e Silva, 1969b). A theory known as the Charnière effect was proposed in which antihistamines are bound to more than one site on the histamine receptor. No allosterism is implied, however, but rather a "hinge effect" whereby one part of the antagonist molecule would remain firmly bound to the so called "non-specific" site but loosely bound to the histamine binding site. Although high doses of agonist could then displace the part of the antagonist molecule from the histamine binding site, the antihistamine would remain bound to the "non-specific" site (Rocha e Silva, 1969b).

A recent hypothesis by Levitzky (1974) combines allosterism and spare receptors to explain membrane receptor phenomena. The basic premise is that receptors exist in clusters and that binding of drug to one receptor activates not only that receptor, but all of those associated with it in the cluster. There is negative cooperativity, however, and the binding of drug to the other receptors in the cluster is precluded by drug binding to one of the receptors. Low concentrations of drug can therefore activate all of the receptors by binding to a small percentage of the population i.e., one receptor per cluster. The hypothesis is based, however, on the assumption that maximum tissue response can be achieved by activation of a small fraction of the receptor population. This has never been shown experimentally but was actually the conclusion arrived at by Nickerson (1956) to explain the irreversible parallel shift in dose-response curves to β-haloalkylamines. Thus, although the concepts of allosterism are contained in the "negative co-operativity" model, this hypothesis actually holds no advantages over the spare receptor hypothesis and suffers from the same assumptions.

Allosterism has been well defined for many proteins and enzymes (Monod, Changeux and Jacob, 1963) and the application to drug receptors has been discussed (Burgen and Spero, 1968; Nakatsu and Reiffenstein, 1968; Szentivanyi et al., 1970; Moran and Triggle, 1970; Radouco-Thomas, 1971; Kunos, Yong, and Nickerson, 1973; Kalsner, 1973; Kuhnen-Clausen, 1974). It has long been known that the function of active sites on drug receptors can theoretically be influenced by drugs bound to neighborhood or "side receptors" (Koshland, 1960; Belleau, 1965; Wilson, 1967). The ability of certain agonists to become "allosteric effectors" (Monod, Wyman and Changeux, 1965) of drug receptors is the essence of two-site theories like that produced by Moran and Triggle (1970). It has been noted by other investigators that the acetylcholine receptor of the excitable membrane of the electroplax possesses regulatory properties (Podleski and Changeux, 1970; Changeux, 1973; Meunier and Changeux, 1973). Care must be taken, however, when applying techniques primarily utilized in studies on isolated enzymes to the considerably more complicated system of an isolated tissue preparation. Kuhnen-Clausen (1974) noted that the Hill coefficient for the histamine response changed from 1.8 to 1.11 upon treatment of the tissue with dibenamine. It is well known that Hill coefficients greater than unity imply cooperativity (Monod, Changeux and Jacob, 1963 ; Monod, Wyman and Changeux, 1965; Podleski and Changeux, 1970). The difficulty comes in assessing where this cooperativity lies, whether at the receptor level or further on in the excitation-contraction mechanism. The change in Hill coefficient observed with dibenamine therefore cannot be regarded necessarily as a receptor effect and provides little information about whether the histamine H_1 receptor has allosteric properties.

Another method of describing possible allosterism in receptors is by a two-state model. There are subtle differences between the concept of allosterism in this scheme and in the two-site models already discussed.

I. TWO-STATE THEORIES AS APPLIED TO DRUG RECEPTORS

A simple model first proposed by Monod, Wyman and Changeux (1965) to describe allosteric proteins and applied, by others, to drug receptors (Karlin, 1967; Edelstein, 1972; Thron, 1973; Rang, 1973; Colquhoun, 1973) provides another description of how β-haloalkylamines could function as allosteric effectors of drug receptors. This two-state model describes an existing equilibrium between two forms of the receptor; a form R which is inactive and a form R^* which is termed active and results in contraction (Rang, 1973). This equilibrium is characterized by a term "L" called the "allosteric constant" (Monod _et al_., 1965) and is defined by the ratio of the concentrations of the R and R^* states ($[R]/[R^*]$). With no drug present, the equilibrium normally lies heavily toward the R form. Histamine would cause contraction by preferentially binding to the active state receptor R^* effectively removing R^* thus altering the equilibrium and shifting it toward the formation of more R^*. Response depends on $[R^*]$ whether combined with drug or not. Partial agonists are defined by this hypothesis as agents with differential affinities for R and R^*, still favoring R^* but not to the extent that a full agonist does. Antagonists prevent agonists from perturbing the equilibrium between R and R^* by binding equally well to the R and R^* state thereby preventing activation by agonist. It is theoretically possible, in this scheme, for an antagonist to possess "negative efficacy" by binding preferentially to the R state.

This model is often termed "co-operative" but this should not be confused with the "induced fit" type of cooperativity as described by Koshland and Neet (1968). While the latter theory implies the actual facilitation of drug binding to one site on the receptor by binding of an allosteric effector to another site, the two-state model is cooperative by virtue of the fact that binding of agonist to R^* shifts the equilibrium toward the production of more R^*. This generates more binding sites for the agonist and leads to further drug binding (Rang, 1973).

By preferentially stabilizing the R form of the receptor, β-haloalkylamines would increase the allosteric constant "L" and thus cause a parallel shift in the dose response curve to full agonists, and a depression in maximum for partial agonists. In this regard, β-haloalkylamines could be thought of as allosteric effectors of the H_1 receptor. Depression of the maximum response results if the blocking agent binds to a site at or near the receptor to change the binding properties of histamine. A theoretical treatment of how changes in the allosteric constant and histamine binding properties could cause these effects is presented in Appendix I.

It should be noted that the two-state model, described here, predicts kinetics and equations virtually identical to those already derived for drug receptor interactions (Colquhoun, (1973) and at present, insufficient data is available to test this hypothesis fully.

J. RECEPTOR PROTECTION AND β-HALOALKYLAMINES

It has been demonstrated that agonists and competitive antagonists, acting on the α-adrenergic receptor, protect against irreversible blockade by β-haloalkylamines (Nickerson and Gump, 1949; Nickerson et al., 1953; Graham, 1962; Innes, 1962a, 1962b; Waud, 1962). This data has been taken as evidence that β-haloalkylamines interact at the same site as do agonists in the adrenergic receptor system (Belleau, 1958; Graham, 1962; Triggle, 1964).

With respect to the histamine receptor, Furchgott (1954) demonstrated some protection against dibenamine blockade in rabbit aorta with histamine while Nickerson (1956) could fully protect against SY28 blockade in guinea pig ileum with diphenhydramine and partially with histamine. Innes (1962a, 1962b) used high concentrations of histamine to demonstrate protection against phenoxybenzamine blockade in spleen while Kuhnen-Clausen (1974) observed some protection with histamine in guinea pig ileum. The data, presented in these studies, however, is difficult to analyze and the full extent of the protection is not known. Takagi and coworkers (1972), however, display full dose response curves to histamine and demonstrate good protection against dibenamine blockade with high doses of histamine in taenia caeci.

Numerous difficulties are encountered in the interpretation of receptor protection studies. Dikstein and Sulman (1965) noted that protection by adrenaline in their experiments was highly unpredictable while Moran and coworkers (1967) found noradrenaline to be protecting many non-specific sites as well as the α-receptor. Many other investigators have encountered this protection of non-specific β-haloalkylamine binding sites with the high doses of agents required to show protection (Waud, 1962; Moran _et al_., 1967; May _et al_., 1967; Terner, Cook and Marks, 1971) and have concluded that the α-receptor population is too small, with respect to the non-specific binding sites, to be specifically protected (May _et al_., 1967; Yong and Nickerson, 1973). Although it may be argued that abnormally high concentrations of reversible protecting agents are required to compete with irreversible antagonists, the problem of non-specific protection is a severe handicap to the interpretation of results from these studies. It would be better if it were possible to demonstrate receptor protection by concentrations of agonists in the physiological range but lack of studies in this regard make it difficult to analyze the concept that β-haloalkylamines interact at the agonist binding site. It must be remembered, further, that if protection is observed, this would not necessarily imply a common binding site in the light of the allosteric concept of protein activation. Factors such as desensitization must also be considered in view of recent studies on the metaphilic effect (see section on Desensitization) and other desensitization-alkylation phenomena (Rang and Ritter, 1969; Miledi and Potter, 1972).

K. HISTAMINE DESENSITIZATION

It is well known that prolonged exposure to high concentrations of histamine renders a tissue temporarily insensitive to low concentrations of histamine for a period of time thereafter. This phenomenon is known as histamine desensitization and currently, there is controversy over whether this is a receptor effect or an effect on the excitation-contraction mechanism. Desensitization to some histamine-like agonists has been discovered to be simply a tachyphylaxis due to the ability of the agent to deplete histamine stores (Halpern, 1956). Ambrus and coworkers (1951) have established that desensitization is not due to a build-up of products from degradation by histaminase acting as reversible antagonists. Some mechanisms proposed suggest an increase in both the catabolism and excretion of histamine as the cause of desensitization (Ambrus _et al_., 1972) but the observation of the phenomenon in isolated tissues makes this unlikely. There is, instead, growing evidence that desensitization to histamine occurs at the receptor level. A recent hypothesis by Ambrus and coworkers proposes that a dual receptor system of "acting" and "storing" receptors is responsible for the effect.

A serious difficulty, in the study of this phenomenon in guinea pig longitudinal smooth muscle is the non-specific aspect of the desensitization produced by histamine (Gosselin _et al_., 1972). While some investigators can induce desensitization to acetylcholine with no loss in sensitivity to histamine (Paton, 1967), the reverse procedure of desensitization to histamine without concomitant effects on acetylcholine cannot be achieved (Bown _et al_., 1973). The general opinion, at this time, is that desensitization to histamine is partly non-specific (Cantoni and Eastman, 1961; Paton, 1961) and partly specific (Schild, 1973a). It was observed by Schild (1973b) that high concentrations of calcium protected against histamine desensitization and this led to the hypothesis that the loss in histamine sensitivity could be related to exhaustion of calcium stores. This hypothesis would be in accordance with that which states that receptors cause contraction by raising the level of free intracellular calcium for contraction by either liberating calcium from a bound site or increasing the permeability to extracellular calcium (Durbin and Jenkinson, 1961; Edman and Schild, 1962; Daniel, 1965; Van Breeman and Lesser, 1971). It was found, however, that desensitization to histamine persisted even after exposure to large concentrations of calcium theoretically capable of replenishing the exhausted calcium stores (Schild, 1973b).

There is much better evidence that desensitization of the cholinergic response is a receptor phenomenon (Rang and Ritter, 1970a). It has been observed that some reversible and irreversible antagonists become much better blocking agents when applied during or shortly after exposure to carbachol or succinylcholine than when applied beforehand (Flacke and Yeoh, 1968; Rang and Ritter, 1969). This phenomenon has since been termed the "metaphilic effect" and provides good experimental evidence that desensitization involves a temporary alteration in receptor conformation (Rang and Ritter, 1969). Because the ability of agonists to cause the metaphilic effect parallels their ability to generate desensitization, the two phenomenon are thought to reflect the same modification (Rang and Ritter, 1970b). Miledi and

Potter (1972) found that desensitization to cholinergic agonists in skeletal muscle greatly reduced irreversible binding of α-bungarotoxin, further implying a receptor mediated mechanism. Extensive studies with calcium in longitudinal smooth muscle also led Chang and Triggle (1973) to conclude that desensitization to cholinergic agents was due to conformational changes at the receptor level.

L. GENERAL REMARKS

In providing an introduction to the work discussed in this thesis, it has been necessary to review a wide range of studies of the receptor. It should be stressed that the reason for the parallel shift in dose response curves obtained by irreversible agents is still a matter for debate. Much more important is the fact that if this irreversible shift is the result of allosteric effects on the receptor, this would do much to reconcile the present differences, perhaps artificial, between enzyme kinetics and action, and drug-receptor kinetics and activation. Irreversible agents possess unique characteristics for the study of receptor mechanisms and thus become useful tools in this regard.

At present, it is unresolved whether these irreversible agents bind directly to the receptor active site or to a secondary site linked to the receptor active site in an obligatory manner. If there are two binding sites for these antagonists, it is unclear whether or not one is the histamine binding site. More complex than a problem in semantics, is the question of whether or not we are dealing with two sites on the same receptor macromolecule or two forms of the receptor in equilibrium. It is also uncertain whether effects such as desensitization originate primarily from conformational changes in the receptor or from effects in the excitation-contraction coupling mechanism. The work discussed in the ensuing pages represents an attempt to answer some of these questions.

II. METHODS

A. ISOLATED TISSUE PREPARATION

Adult male guinea pigs weighing 200-400 g were killed by a blow on the head, and the terminal ileum was excised, cleaned of contents and immediately placed in Tyrode solution (Tyrode, 1910) the composition of which is shown in Appendix II. The ileum was cut into three-centimeter segments and the longitudinal muscle layer removed by careful swabbing with cotton (Rang, 1964). The longitudinal muscle strips were suspended in Tyrode solution at 37°C, gassed with 95% oxygen - 5% carbon dioxide (pH 7.2 - 7.4), under a resting tension of 300-500 mg. Contractions were recorded isotonically using a Hewlett-Packard linear motion transducer model 1000-7DCDT connected to a Grass model 5P polygraph (Fig. 18).

Each tissue was allowed to equilibrate for one hour at 37°C and the bathing medium was changed every thirty minutes during this period.

A dose-response curve to histamine was then obtained. Cumulative dose-response curves were found to be identical to dose-response curves obtained with wash periods between doses of histamine (see Chapter 3 - Section A) providing that the increments in dosage were added in a rapid and reproducible manner. Some studies reported here utilized cumulative dose-response curves while others did not, but the curve after blockade was always obtained by the same method as the control curve for all tissues in a given experiment.

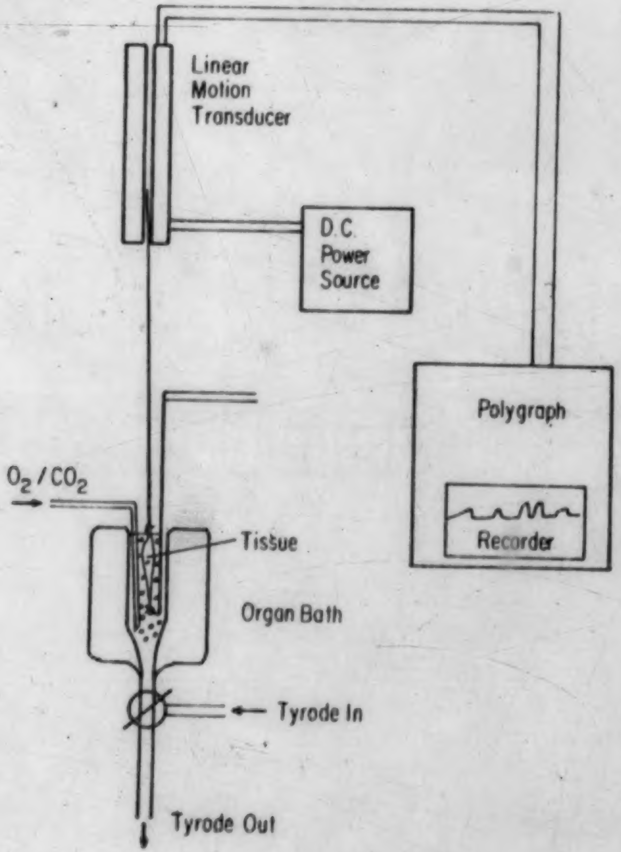

Linear
Motion
Transducer
D.C.
Power
Source
Polygraph
Recorder
O_2/CO_2
Tissue
Organ Bath
Tyrode In
Tyrode Out

Figure 18. Schematic diagram of apparatus used for studies of the contraction of guinea pig ileum and longitudinal smooth muscle strips from the guinea pig ileum.

B. BLOCKADE BY β-HALOALKYLAMINES

The β-haloalkylamines were dissolved in saline and stored for 15-30 minutes at room temperature before use as studies have shown a maximum concentration of aziridinium ion being present 15-40 minutes after dissolution (Graham, 1957; Gill and Rang, 1966; Hirst and Jackson, 1972). Aliquots of the solution of β-haloalkylamine were then added to the tissue bathing medium and removed by wash after a designated period of time. In experiments utilizing N,N,-dimethyl-2-bromophenylethylamine (DMPEA) (Fig. 19) a compound known to have a somewhat faster rate of cyclization than most other β-haloalkylamines (Kimelberg and Triggle, 1965) the solution was used 5 minutes after the drug was dissolved.

The thiosulphate ion has long been known to produce a complex with the aziridinium ion (see Fig. 6). The first description of this reaction was provided by Bünte (1874) and the resulting pharmacologically inactive complex subsequently called a "Bünte salt". As many results indicate that the shift in dose-response curve caused by β-haloalkylamines is the result of a covalent antagonist-receptor complex, and since the presence of unbound aziridinium ion tended to obscure the shift in the dose-response curve (see Chapter 3 - Section C) experiments designed to study this effect utilized thiosulphate in the wash medium. It became apparent, however, that the depression of the maximum response produced by these agents was most likely a result of non-covalent binding of aziridinium ion (see Chapter 3 - Section G) thus in studies on the depression of the dose-response curve, thiosulphate was not present in the wash. In experiments where the difference in the magnitude of depression of the maximum response between two tissues was required, thiosulphate tended to abolish this difference by scavenging aziridinium ion, thereby removing the blockade produced by this species.

C. REVERSAL OF BLOCKADE WITH BSA/THIOSULPHATE

i) Depression of Maximum Response

In some tissues in which the maximum response was depressed by phenoxybenzamine, washing was continued for up to 18 hours in an attempt to detect any reversal of blockade. Concurrently, 0.5% Bovine Serum Albumin (BSA) and 10^{-3}M sodium thiosulphate were added to the wash medium for other tissues and wash carried out as described above. At various times, the maximum response was tested for both tissues using a supramaximal dose of histamine (10^{-4}M). In the case of the BSA-washed tissues, the BSA was removed by wash with a constant stream of Tyrode solution for 60 seconds before the testing of response, as the protein is known to bind histamine with facility (Durand et al., 1971). Each pair of tissues was tested for response no more than 4 times throughout the wash period. The procedure is shown schematically in Fig. 20.

The maximum height of contraction obtained was calculated as a percentage of the tissue maximum response and the difference from 100 designated as "percent blockade". The data for recovery of response were plotted as the logarithm (base 10) of blockade against time, to yield a straight line relationship, as expected for a first order reaction (Kimelberg and Triggle, 1965). In many cases the blockade was profound and no recovery could be detected until after 1 or 2 hours

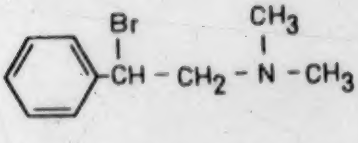

Br
CH3
CH - CH2 - N - CH3

Figure 19. N,N,-dimethyl-2-bromophenylethylamine (DMPEA)

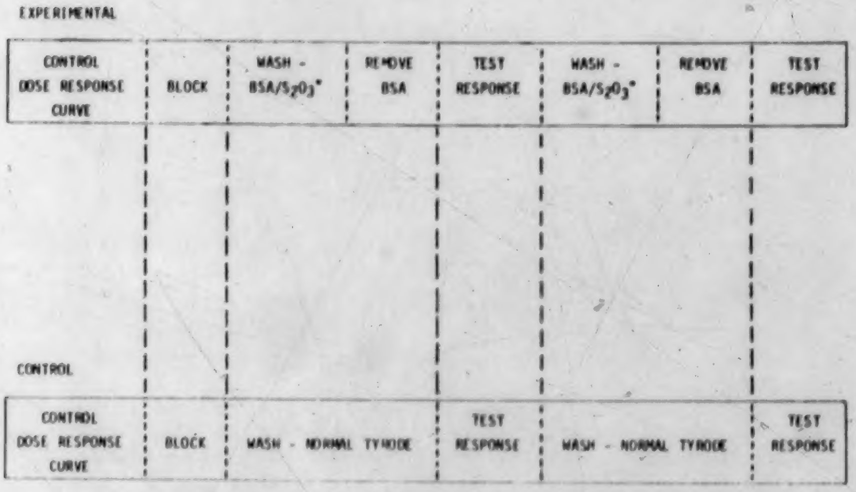

EXPERIMENTAL
CONTROL DOSE RESPONSE CURVE
BLOCK
WASH - BSA/S2O3=
REMOVE BSA
TEST RESPONSE
WASH - BSA/S2O3=
REMOVE BSA
TEST RESPONSE
CONTROL
CONTROL DOSE RESPONSE CURVE
BLOCK
WASH - NORMAL TYRODE
TEST RESPONSE
WASH - NORMAL TYRODE
TEST RESPONSE

Figure 20. Schematic diagram of procedure for experiments utilizing BSA/thiosulphate wash (see text - Chapter II - Section C)

of wash. The recovery for these tissues was then corrected so that the straight line defined by the log (Blockade) against time relationship intersected the ordinate at log (Blockade) = 2 (Blockade equals 100% at time 0).

ii) Parallel Shift of the Dose-Response Curve

In some experiments, lower concentrations of phenoxybenzamine were employed to yield a parallel shift in the dose-response curve with no depression of maximum response. The BSA/thiosulphate treatment was identical to that used in the study of depression of maximum in an attempt to detect any reversal of the parallel shift. The tissues were paired as before with differences in parallel shift being noted in $\log_{10}$ units.

D. REVERSAL OF BLOCKADE BY MERCAPTOPYRUVATE

Mercaptopyruvate was utilized in attempts to detect possible receptor effects of phenoxybenzamine originating from an intracellular locus (Graham *et al.*, 1968; Graham *et al.*, 1971; Mottram, 1974), as exogenously administrated thiosulphate ion is known to penetrate cells very poorly (Cardozo and Edelman, 1952).

Mercaptopyruvate (10^{-3}M),an agent capable of entering cells and reacting with aziridinium ion (Fästh and Sorbo, 1973), was incubated with longitudinal smooth muscle for a period of 30 minutes after which the portion which had not been taken up by the tissue was removed by wash. This agent is known to produce intracellular thiosulphate ion *in vivo* (Fästh and Sorbo, 1973) and in rat liver homogenate (Sorbo, 1957*a*) by participating in an enzymic transfer of the mercaptopyruvate sulphur. The mercaptopyruvate is also known to react with aziridinium ion to the same extent as thiosulphate thus it is not immediately obvious whether intracellular enzymically-produced thiosulphate or intracellular mercaptopyruvate itself is available to react with intracellular β-haloalkylamine. The tissues treated with mercaptopyruvate as well as a control tissues were washed for 1 hour and then exposed to phenoxybenzamine (10^{-5}M), and the reversal of the depression of maximum response caused by the β-haloalkylamine, was observed over a 3 hour wash period as in the BSA/thiosulphate studies described in Methods - Section C. The procedure is outlined schematically in Fig. 21.

In order to detect any intracellularly generated thiosulphate, tissues exposed to mercaptopyruvate for thirty minutes were homogenized with a Polytron® (Kinematica GMBH) 20 seconds, filtered and the filtrate assayed for thiosulphate with the colorimetric method described by Sorbo (1957b). To 5 ml of the filtrate, 0.5 ml 0.1 N potassium cyanide and 0.3 ml 0.1 N cupric chloride were added, and the mixture stirred on a vortex mixer. Then, 0.5 ml of ferric nitrate reagent (100 g $Fe(NO_3)_3 \cdot 9H_2O$ and 200 ml 65% nitric acid plus distilled water to 1000 ml) was added and the solution stirred again. A blank was prepared in the same manner from filtrate of homogenized tissue not treated with mercaptopyruvate and the absorption at 460 nm determined in 1 cm cuvettes on a Hitachi Perkin-Elmer 139 UV-Vis spectrophotometer.

E. ASSAY OF AZIRIDINIUM ION

The most commonly used method for determining β-haloalkylamine cyclization rates is to back titrate thiosulphate ion, not consumed by

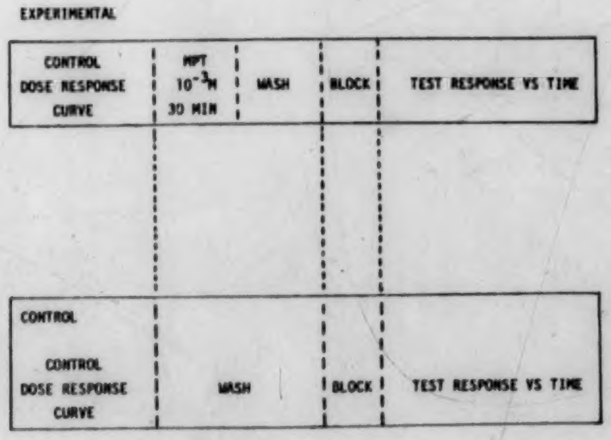
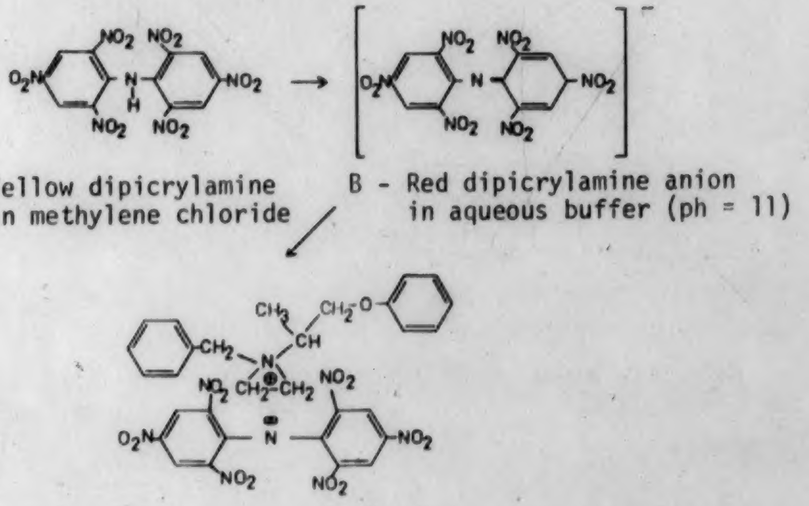

EXPERIMENTAL
CONTROL DOSE RESPONSE CURVE
MPT 10⁻³M 30 MIN
WASH
BLOCK
TEST RESPONSE VS TIME
CONTROL
CONTROL DOSE RESPONSE CURVE
WASH
BLOCK
TEST RESPONSE VS TIME

Figure 21. Schematic diagram of procedure for experiments utilizing mercaptopyruvate pretreatment (see text - Chapter II - Section D).


NO2
O2N
N
H
NO2
NO2
NO2
NO2
NO2
CH3
CH2-O
CH2
CH
N
CH2-CH2

A - Yellow dipicrylamine in methylene chloride

B - Red dipicrylamine anion in aqueous buffer (ph = 11)

C - Dipicrylamine anion - phenoxybenzamine aziridinium ion - "ion pair" (red)

Figure 22. Formation of ion pair between aziridinium ion for phenoxybenzamine and dipicrylamine anion in methylene chloride (see text - Chapter II - Section E).

aziridinium ion, with iodine. This is unsuitable for the determination of the cyclization rate of phenoxybenzamine as the low solubility of this drug does not permit adequate concentrations to be attained for this procedure. Schill and Danielsson (1959) found that hexanitrodiphenylamine (dipicrylamine) formed an ion pair with quaternary ammonium compounds and that many of these complexes could be extracted into organic solvent. The complexes have high molar extinction coefficients thus small amounts of quaternary ammonium compounds could be determined spectrophotometrically. The method has been successfully extended, by Fästh and Sorbo (1973), to the determination of aziridinium ion concentrations for various nitrogen mustards and is based on the principle that the positively charged aziridinium ion forms the ion pair with the dipicrylamine anion. The ion pair is then extracted into methylene chloride and the absorbance determined at 420 nm (see Fig. 22).

A solution of phenoxybenzamine (10^{-5}M) was prepared in phosphate buffer (100 ml) at pH 7.4 (Appendix II) and incubated at 37°C. Aliquots of 10 ml were removed at various times, 0.1 N NaOH (2 ml) added, followed by 5 ml ice cold dipicrylamine reagent (10 mg dipicrylamine in 100 ml methylene chloride) and the mixture was stirred on a vortex mixer. Three ml of the organic phase were removed, placed in a 1 cm cuvette and the absorbance determined on a Hitachi Perkin-Elmer 139 UV-Vis spectrophotometer. A blank consisting of buffer only was used for comparison.

F. ESTIMATION OF RECEPTOR RESERVE

Waud (1968) has described a method by which the apparent fraction of receptors irreversibly blocked can be calculated from a plot comparing reciprocals of equiactive doses of agonist before and after blockade. This procedure relates the magnitude of the parallel shift induced to the percentage of receptors inactivated. Thus the largest shift obtainable with retention of maximum response reflects the blockade of the highest percentage of receptors not required for the production of the maximum response; i.e., the "receptor reserve". The equation describing the "double reciprocal plot" used to calculate this percentage is derived from Clark's (1933) original equation describing occupation of receptors by drugs.

$$y = \frac{A}{A + K_a}$$

q = proportion of receptors irreversibly blocked

Therefore response after blockade is proportional to (1 - q). After blockade:

$$y' = \frac{A'}{A' + K_a}(1 - q)$$

When equiactive doses are compared and assuming equal response means equal receptor occupation by agonist:

$$y' = y$$

$$\frac{A}{A + K_a} = \frac{A'}{A' + K_a}(1 - q)$$

A' is a dose equiactive to A.

$$\frac{K_a + A'q}{A'} = \frac{K_a - qK_a}{A}$$

$$\frac{1}{A} = \frac{1}{A'}\cdot\left(\frac{1}{1 - q}\right) + \frac{1}{K_a}\cdot\left(\frac{q}{1 - q}\right)$$

Thus a plot of $\frac{1}{A}$ against $\frac{1}{A'}$ yields a straight line with slope equal to $\frac{1}{1 - q}$. When dealing with preparations in which the maximum shift before depression of maximum response has been induced, the receptor reserve equals (slope - 1)/slope.

G. PRETREATMENT WITH N,N,-DIMETHYL-2-BROMOPHENYLETHYLAMINE (DMPEA)

For some tissues, a control dose response curve to histamine was obtained, DMPEA (10^{-5}M) added to the bathing medium for a period of 5 minutes, and a second dose response curve determined after removal of the DMPEA by wash. Phenoxybenzamine (10^{-5}M) was then added for 5 minutes, the tissue washed for 1 to 2 hours, and a third dose-response curve obtained. Concurrently, tissues not pretreated with DMPEA were subjected to phenoxybenzamine bloackade in the same manner as described above. The procedure is shown schematically in Fig. 23.

The means of the maxima from the tissues pretreated and not pretreated with DMPEA were compared and analyzed by Student's **t-test**.

H. DESENSITIZATION

Paired tissues were employed to determine the effect of desensitization on the degree of blockade produced by phenoxybenzamine. Control tissues were exposed to phenoxybenzamine (2 x 10^{-6}M) as described previously while experimental tissues were desensitized to histamine before treatment with alkylating agent. Desensitization was induced by addition of 10^{-4}M histamine, to the bathing medium, for varying lengths of time. The agonist was then removed by wash and the dose ratio (*vide infra*) determined by inducing contractions with doses of histamine in the ED40 to ED60 range. Upon completion of the dose-ratio determination, the tissue was immediately subjected to phenoxybenzamine treatment. Both tissues were washed under identical conditions, thiosulphate ion being present when the parallel shift of the dose response curve was being investigated, and absent when the experiment dealt with depression of the maximum response. The response to histamine was then determined after a wash period of at least 1 hour. (It was found, from studies on control tissues, that all effects of desensitization had disappeared after this time.)

This methodology differs somewhat from that of Rang and Ritter (1970*b*) who also studied the effects of desensitization on irreversible blockade but in cholinergic preparations. They allowed recovery after their measurement of desensitization, induced the effect again and treated the tissue with alkylating agent after waiting a period of time equal to that which had elapsed before the measurement of the extent of desensitization. Thus they did not have to alter the induced desensitization by subjecting the tissue to the doses of histamine used to measure the dose ratio. It must be assumed, however, that the same amount of desensitization will be induced the second time as was induced the first, an assumption which appears to be valid for the cholinergic receptors of the chick biventer cervicus muscle, but not for the longitudinal smooth muscle of the ileum. It was found that the second exposure to high doses of histamine produced a desensitization of unpredictable magnitude. As the actual quantitation of the extent of desensitization is not of importance in itself and as the method of Rang and Ritter (1970b) was shown to be impracticable in ileum, the blocking agent was added after the measurement of the dose ratio was carried out. As discussed in Chapter I, the lack of specificity of the desensitization makes quantitation of this effect difficult. The response to acetylcholine was tested in some tissues that were previously desensitized to histamine in an attempt to measure the extent of the non-specific desensitization.

Highly desensitized tissues do not respond to doses of histamine previously capable of producing a maximum response thus it is possible that such a concentration of histamine could be present, but undetected, in the organ bath during the exposure to alkylating agent. Thus this residual histamine (from the dose of histamine used to cause desensitization) theoretically could protect receptors and cause an effect on alkylation which could erroneously be attributed to the desensitization. To guard against this, some tissues were exposed to phenoxybenzamine in the presence of the highest concentration of histamine which would be undetectable at maximum desensitization, and the effects on blockade noted. The procedures are shown schematically in Fig. 24.

The difference in either the shift (in log units) or depression between the desensitized and non-desensitized tissues was then determined and the significance tested with a paired t-test. The measured differences in blockade were also correlated with the extent of desensitization which was defined by a term ρ. The derivation of this quantity was first described by Paton (1961).

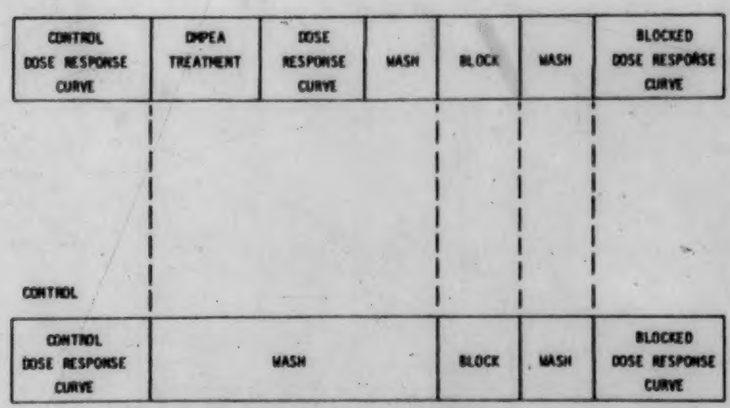

CONTROL DOSE RESPONSE CURVE
DMPEA TREATMENT
DOSE RESPONSE CURVE
WASH
BLOCK
WASH
BLOCKED DOSE RESPONSE CURVE
CONTROL
CONTROL DOSE RESPONSE CURVE
WASH
BLOCK
WASH
BLOCKED DOSE RESPONSE CURVE

Figure 23. Schematic diagram of procedure for DMPEA pretreatment studies (see text - Chapter II - Section G)

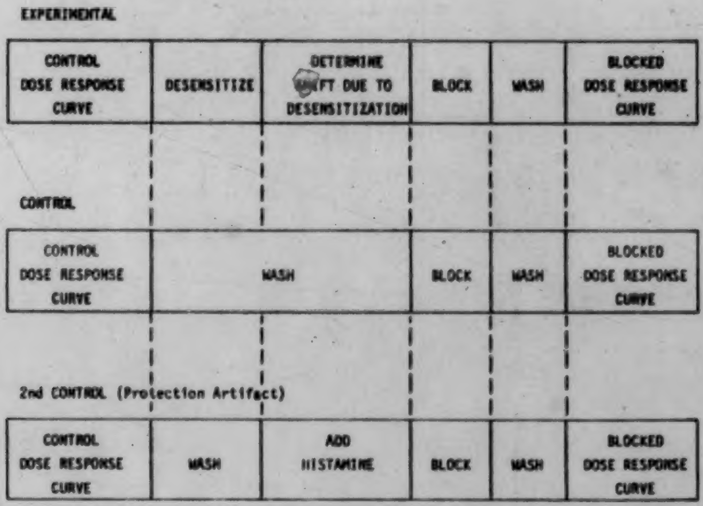

EXPERIMENTAL
CONTROL DOSE RESPONSE CURVE
DESENSITIZE
DETERMINE SHIFT DUE TO DESENSITIZATION
BLOCK
WASH
BLOCKED DOSE RESPONSE CURVE
CONTROL
CONTROL DOSE RESPONSE CURVE
WASH
BLOCK
WASH
BLOCKED DOSE RESPONSE CURVE
2nd CONTROL (Protection Artifact)
CONTROL DOSE RESPONSE CURVE
WASH
ADD HISTAMINE
BLOCK
WASH
BLOCKED DOSE RESPONSE CURVE

Figure 24. Schematic diagram of procedure for studies on the effect of desensitization on blockade by phenoxybenzamine (see text - Chapter II - Section H)

ρ = the proportion of receptors which cannot be activated by agonist to produce response i.e., proportion desensitized.

$(1 - \rho)$ = the proportion of receptors available for combination with agonist to produce response.

DR = dose ratio = the ratio that the dose of agonist must be increased by in order to achieve a response, in the presence of the desensitization, equal to some standard response before desensitization.

$$DR = \frac{1}{1 - \rho} \quad \ldots\ldots \text{(Paton, 1961)}$$

$$\rho = \frac{DR-1}{DR}$$

I. RECEPTOR PROTECTION

i) Protection Against Blockade

In receptor protection experiments, the protecting agent was added to the bathing medium for varying lengths of time, depending on the agent, before and during exposure to phenoxybenzamine. In studies on the protection by full or partial agonists, the irreversible blocking agent was added to the medium 20 seconds after the agonist had reached a plateau in the contraction tracing indicating a constant (generally maximal) response. Although studies by other workers often utilized longer periods of incubation of agonist with the tissue before the addition of antagonist, it was felt that in our studies this would have introduced the variable of desensitization, already noted to have effects on alkylation by phenoxybenzamine. Protection with the reversible antagonist diphenhydramine required an exposure of at least 5 minutes before addition of phenoxybenzamine as studies have indicated that the attainment of an equilibrium between this agent and the receptor population requires this time period (Cook and Krueger, 1975). In view of experiments indicating the possible continuation of the alkylation process after the removal of antagonist (see Chapter III - Section Q), the protecting agent was reintroduced into the bathing medium for a period of 30 minutes after incubation with phenoxybenzamine (despite the risk of desensitization and consequent alteration of blockade) and comparison made to tissues which were not re-exposed to the protecting agent. It was found that the longer periods of incubation with protecting agent had little effect on the blockade produced by the alkylating agent.

In studies concerning the depression of the maximum response, the tissues were exposed to phenoxybenzamine (2×10^{-6}M) for 3 minutes and washed for 2 hours with Tyrode solution containing no thiosulphate ion. In experiments dealing with the shift induced in the dose response curve, the tissue was exposed to phenoxybenzamine (10^{-6}M) for 3 minutes and washed for 2 hours with Tyrode solution containing thiosulphate ion (10^{-3}M).

Paired tissues (selected at random) were used to study differences between antagonism of control and protected preparations. One tissue was protected, both exposed to phenoxybenzamine and both washed for the same period of time before a second dose-response curve was determined. The sequence is shown schematically in Fig. 25. After the wash period, the extent of depression of maximum or shift in dose response curve was determined and the differences between control and protected tissue analyzed by a paired t-test.

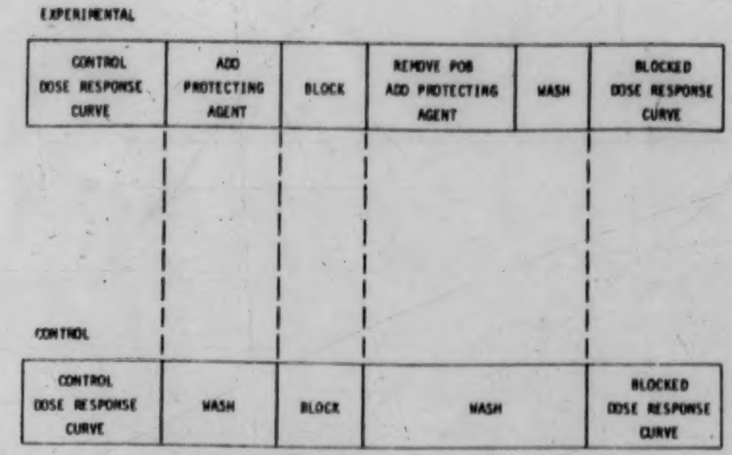

EXPERIMENTAL
CONTROL DOSE RESPONSE CURVE
ADD PROTECTING AGENT
BLOCK
REMOVE POB ADD PROTECTING AGENT
WASH
BLOCKED DOSE RESPONSE CURVE
CONTROL
CONTROL DOSE RESPONSE CURVE
WASH
BLOCK
WASH
BLOCKED DOSE RESPONSE CURVE

Figure 25. Schematic diagram of procedure for receptor-protection experiments (see text - Chapter II - Section I (i)).

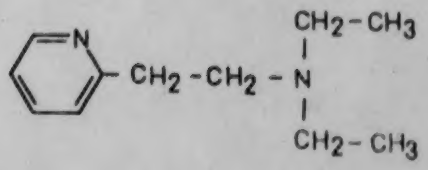


Figure 26. N,N diethyl-2-ethylaminopyridine (Et_2pyretamine)

ii) Increase in Blockade During Wash

It had been noted that, in some tissues treated with phenoxybenzamine and washed with Tyrode which did not contain sodium thiosulphate, the magnitude of the blockade increased over the wash period. It thus seemed possible that significant amounts of uncyclized phenoxybenzamine or phenoxybenzamine-aziridinium ion was binding to nonspecific sites on the cell membrane during the initial exposure of the antagonist, and functioning as a pool of β-haloalkylamine from which further binding to receptors could be achieved. Phenoxybenzamine was therefore dissolved in 0.1 N HCl (to retard cyclization and provide more uncyclized antagonist), added to the bathing medium (5×10^{-6}M) for 5 minutes, and removed by wash. A dose-response curve to histamine was then determined after 30 minutes of wash with Tyrode which did not contain sodium thiosulphate, and again after a wash period of 2 hours. The differences in the magnitude of blockade were then analyzed by a paired t-test.

iii) pA_2 Measurement for the Partial Agonist Et_2pyretamine

Schild (1947) outlined a method for determining whether two drugs act at the same receptor. The procedure yields a term pA_x defined as the negative $\log_{10}$ of the concentration of antagonist at which the ratio of equiactive doses of agonist, in the presence and absence of antagonist, is "x". Thus, two agonists providing identical pA_2, pA_5 and pA_{10} values for any given reversible antagonist, would be defined as acting on the same receptor. The agent Et_2pyretamine (N,N-diethyl-2-ethylaminopyridine, Fig. 26) has been described as a partial agonist for the H_1 receptor (Walter et al., 1941; Hunt and

Fosbinder, 1942) but as no pA_x data was readily availabe, the pA_2 for diphenhydramine was determined for this agent and for histamine.

An ED50 dose for both agonists was determined and then an arbitary concentration of diphenhydramine was allowed to equilibrate with the tissue for at least 5 minutes. Twice the ED50 dose (i.e., $x = 2$) was then utilized to elicit contraction and the preparation washed free of all drugs. If twice the ED50 dose of agonist caused a contraction of smaller magnitude than the ED50 concentration before the presence of reversible antagonist, then the procedure was repeated with a smaller concentration of diphenhydramine. If twice the ED50 concentration of histamine produced a larger contraction than the ED50 did beforehand, the procedure was repeated with a larger dose of reversible antagonist.

A plot was then constructed of the percent of original ED50 contraction produced by twice the dose of histamine in the presence of antagonist as the ordinate and the corresponding concentrations of diphenhydramine as the abscissa. The value on the abscissa corresponding to 100% of initial ED50 contraction on the ordinate (as determined by a straight line joining the 2 points), represents the pA_2.

iv) Agonist-Antagonist Chemical Interactions

It is well known that β-haloalkylamines are able to alkylate electrophilic chemical groups and thus the possibility of reactions occurring between the protecting agents and the blocking agent must be considered. Fruton and coworkers (1946) have shown that nitrogen mustards can form the pyridinium ion and imidazolinium ion with pyridine and imidazole respectively (see Fig. 27) thus there is the possibility of reactions between phenoxybenzamine and either Et_2pyretamine or histamine. Such a chemical antagonism would reduce the amount of blocking agent and give the misleading impression that receptor protection was taking place. Because of this possibility, the amount of aziridinium ion formed after 3 minutes under physiological conditions (pH 7.4, at 37°C) was assayed in the presence and absence of the various protecting agents. The extent of the interaction between pyridine, imidazole and aziridinium ion was not obvious from the study by Fruton and coworkers (1946) as the reactions were carried out under extreme conditions of pH and for time-spans of 12 hours. Thus, in control experiments, phenoxybenzamine (5×10^{-6}M) was added to 25 ml of buffer (Appendix II) and after a 3 minute time period, a 10 ml aliquot removed and assayed for dipicrylamine anion. In other experiments, various concentrations of Et_2pyretamine or histamine were present in the buffer. A slightly modified version of the dipicrylamine assay described in Methods - Section E was then used to determine the amount of remaining free organic base (either Et_2pyretamine or histamine) in the aqueous buffer. Kertes and Kertes (1956) and Kertes (1956) described a method for the determination of pyridine and other organic bases with dipicrylamine. The aliquots (10 ml) of buffer containing phenoxybenzamine and organic base were brought to pH 11 with NaOH (0.1 N-2 ml) and extracted with methylene chloride (5 ml). At the high pH all of the free organic base entered the organic phase, leaving any charged complexes between the base and phenoxybenzamine (i.e., pyridinium ion) in the aqueous phase. 2 ml Of the methylene chloride phase were then combined with 1 ml of the dipicrylamine

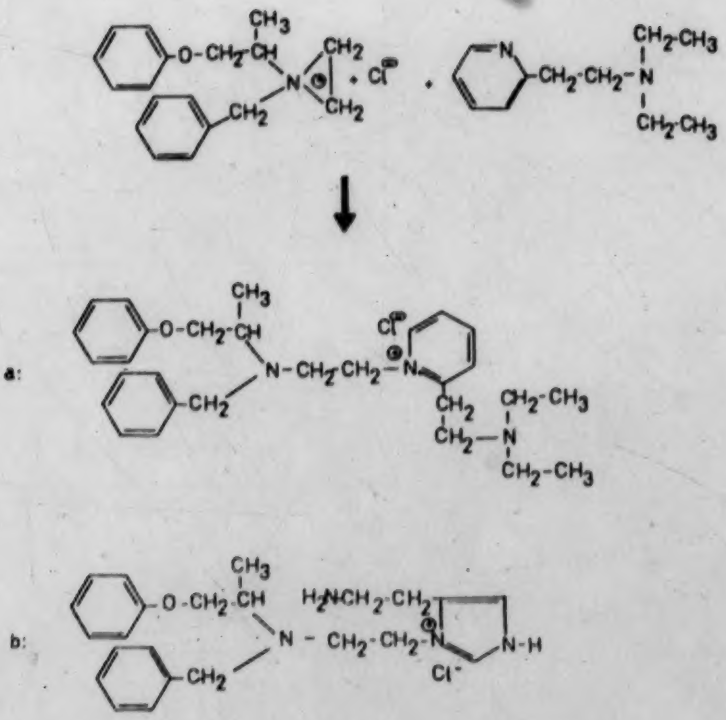


Figure 27. Formation of complexes between phenoxybenzamine and agonists

a: Pyridinimium ion formed from the aziridinium ion of phenoxybenzamine and Et$_2$pyretamine.

b: Imidazolinium (or histamininium) ion formed from the aziridinium ion of phenoxybenzamine and histamine.

reagent (Chapter III - Section E) in a 1 cm cuvette. The organic base becomes a proton acceptor for the dipicrylamine hydrogen and the red dipicrylamine anion is thereby generated. A study by Schill and Danielsson (1959) showed that this anion absorbs at 420 nm. Differences between the amount of free Et_2pyretamine in buffer containing phenoxybenzamine and buffer alone indicate the amount of Et_2 pyretamine reacting with phenoxybenzamine. By relating this fraction to the relative concentrations of Et_2pyretamine and phenoxybenzamine, a measure of the amount of phenoxybenzamine forming the pyridinium ion with Et_2pyretamine, under these conditons, can be determined. The uncyclized phenoxybenzamine is not a strong enough base to cause interference in this assay.

As histamine was a rather poor proton acceptor for dipicrylamine leading to low absorbance values, the amount of aziridinium ion reacting with Et_2pyretamine was determined in the presence and absence of histamine. If histamine were significantly depleting the aziridinium ion, then after 3 minutes, there would be less of this species to react with Et_2pyretamine. The following procedure was therefore employed. Phenoxybenzamine (10^{-5}M) was added to buffer (25 ml) at pH 7 and 37°C in the presence and absence of histamine (10^{-4}M). After 3 minutes, Et_2pyretamine (10^{-5}M) was added to both samples and to a third sample of buffer. 3 Minutes later, all 3 were assayed for free Et_2pyretamine as described. Reaction between histamine and phenoxybenzamine would be reflected by a greater concentration of free Et_2pyretamine in the sample containing phenoxybenzamine and histamine.

The reaction between phenoxybenzamine and various concentrations of thiosulphate was determined. In these experiments, phenoxybenzamine was incubated in buffer as before in the presence and absence of thiosulphate ion. After 3 minutes, the samples were assayed for aziridinium ion as in Chapter II - Section E.

Estimations of the pharmacological effect of this chemical antagonism can be derived by carrying out the experiments to determine blockade in the presence of a concentration of thiosulphate ion found to chemically deplete the β-haloalkylamine aziridinium ion concentration to the same degree as the protecting agent. Paired tissues were used, 1 exposed to blocking agent and 1 to antagonist in the presence of thiosulphate and the difference in blockade recorded.

v) Effect of Histaminase Blockade in Protection Experiments

If the enzyme histaminase were hydrolyzing and thereby depleting the agonist concentration protecting the receptors against β-haloalkylamine blockade, the protecting effect of this agonist would then be underestimated. Such a problem could theoretically occur when comparing the protection provided by the agents Et_2pyretamine and histamine as the latter can be hydrolyzed by histaminase while the former connot (Arunlakshana, Mongar and Schild, 1954).

The ability of histamine to protect receptors, in some experiments, was therefore studied in the presence of the histaminase inhibitor, hydroxylamine (Zeller, 1942). The protection experiments described in Section J.(i) were carried out in the presence of 10^{-5}M hydroxylamine and the resulting protection compared to the studies with no histaminase inhibition by Student's t-test. The possibility of protection by hydroxylamine itself was tested with the appropriate control.

III RESULTS

A. DOSE-RESPONSE CURVES TO HISTAMINE

There is no significant difference between dose-response curves obtained with washing between exposures to agonist and those obtained by sequential addition of cumulative concentrations of agonist (Fig. 28). In all experiments, the dose-response curve after blockade, was determined in the same manner as the control.

B. SPECIFICITY OF BLOCKADE BY β-HALOALKYLAMINES

As antagonists of the β-haloalkylamine type are known to be fairly non-specific (Furchgott, 1954), the selectivity of the blockade was tested in this preparation by observing their effects on the response to a number of agonists.

At the concentrations and exposure times employed in these experiments, the blockade produced by the β-haloalkylamines appeared to be relatively selective for the histamine response. Figure 29 shows the antagonism of the response to histamine by SY14 (2×10^{-7}M, 3 min) while Fig. 30 shows the small concomitant blockade of response to acetylcholine. The responses to potassium chloride (Fig. 31), barium chloride, (Fig. 32) and prostaglandin $F_{2\alpha}$ (Fig. 33) were not significantly altered by this treatment with SY14. When higher concentrations of antagonist were used, such that the response to histamine was all but abolished, the blockade of the acetylcholine response became more apparent. This is shown in Fig. 34 and 35 for phenoxybenzamine, an antagonist known to block the acetylcholine response when used in high concentrations (Furchgott, 1954; Cook, 1971). The parallel shift in the dose response curve to acetylcholine produced by

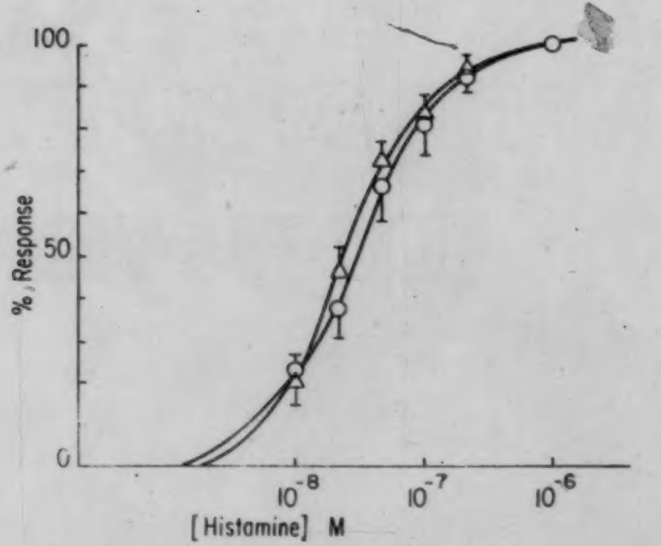


Figure 28. Dose-response curve for histamine. Molar concentration as abscissa, percent maximum response as ordinate. N = 4. Bars represent standard errors.

o——o wash between doses of agonist

Δ——Δ sequential addition of cumulative concentration of agonist

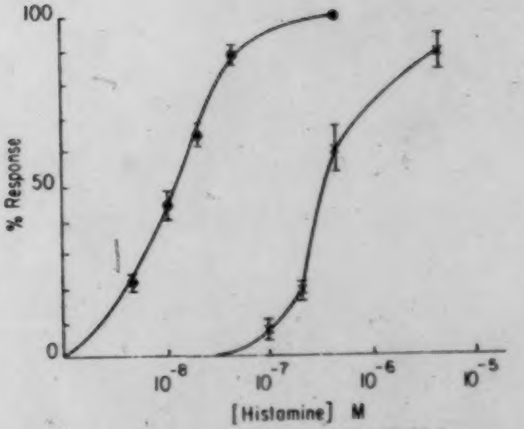


Figure 29. Effect of SY14 on response to histamine. Dose-response curve for histamine. Molar concentration as abscissa, percent maximum response as ordinate. N = 4. Bars represent standard errors.

●—● control dose-response curve for histamine

x—x response to histamine after SY14 (2×10^{-7}M for 3 min)

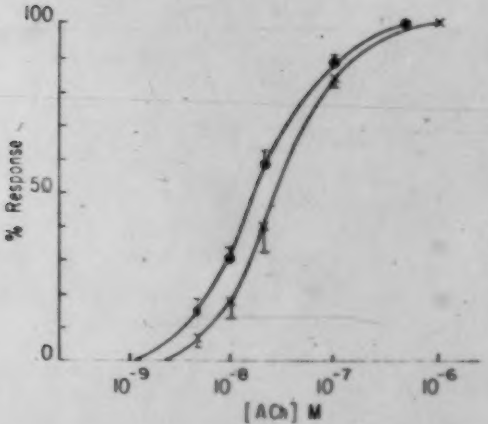


Figure 30. Effect of SY14 on response to acetylcholine. Dose-response curve for acetylcholine. Molar concentration as abscissa, percent maximum response as ordinate. N = 4. Bars represent standard errors.

●—● control dose-response curve for acetylcholine

x—x response to acetylcholine after SY14 (2×10^{-7}M for 3 min)

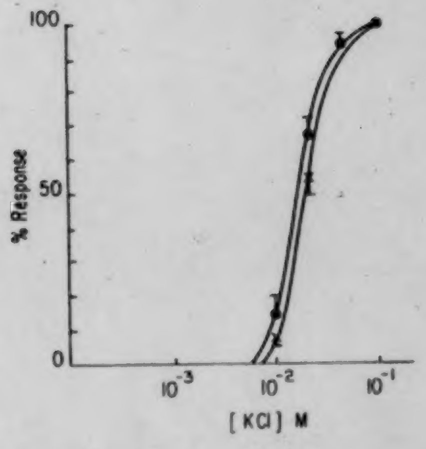


Figure 31. Effect of SY14 on response to potassium chloride. Dose-response curve for potassium chloride. Molar concentration as abscissa, percent maximum response as ordinate. N = 4. Bars represent standard errors.

●—● control dose-response curve for potassium chloride

x—x response to potassium chloride after SY14 (2×10^{-7}M for 3 min)

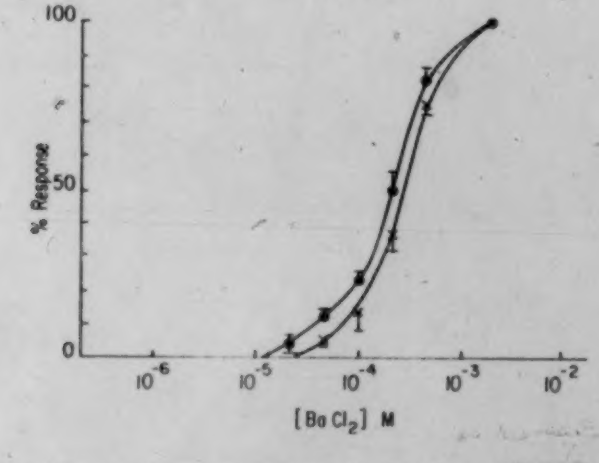


Figure 32. Effect of SY14 on response to barium chloride. Dose response curve for barium chloride. Molar concentration as abscissa, percent maximum response as ordinate. N = 4. Bars represent standard errors.

●—● control dose-response curve for barium chloride

x—x response to barium chloride after SY14 (2×10^{-7}M for 3 min)

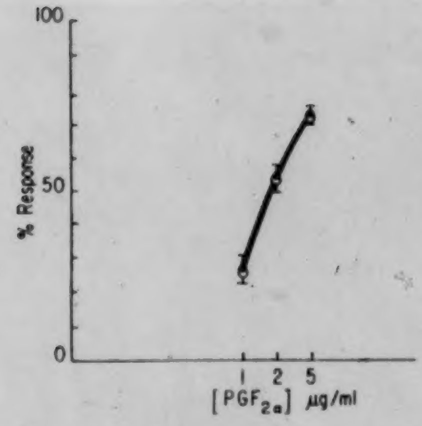


Figure 33. Effect of SY14 on response to prostaglandin $F_{2\alpha}$. Dose response curve for prostaglandin $F_{2\alpha}$. Concentration as abscissa, percent maximum response as ordinate. N = 4. Bars represent standard errors.

•—• control dose response curve to $PGF_{2\alpha}$

o- -o response to $PGF_{2\alpha}$ after SY14 (2 x 10^{-7}M for 3 min)

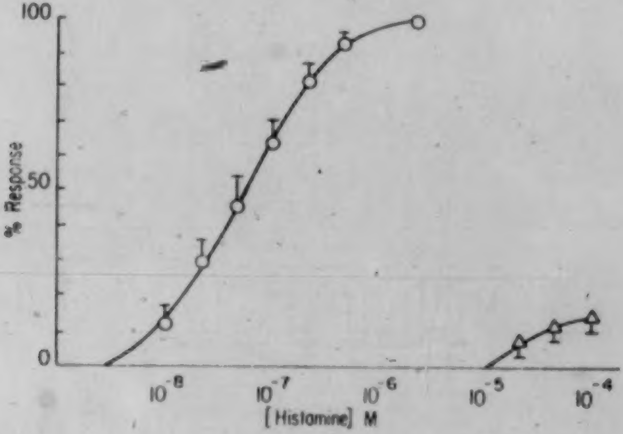


Figure 34. Blockade of histamine response by phenoxybenzamine (2 x 10^{-5}M for 3 min). Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 4. Bars represent standard errors.

o—o control dose response curve

Δ—Δ dose-response curve after phenoxybenzamine

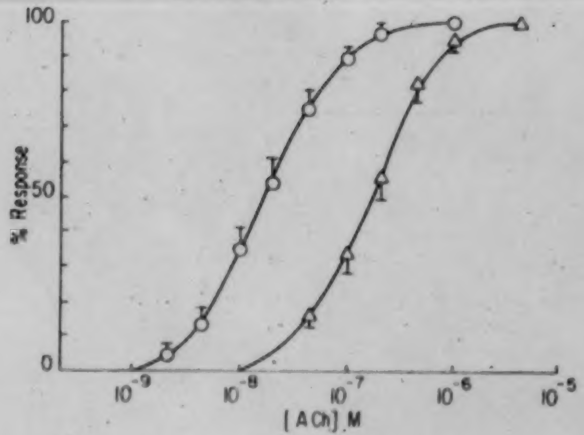


Figure 35. Effect of phenoxybenzamine (2 x 10^{-5}M for 3 min) on response to acetylcholine. Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 4. Bars represent standard errors.

o—o Control dose-response curve

Δ—Δ Dose-resonse curve after phenoxybenzamine

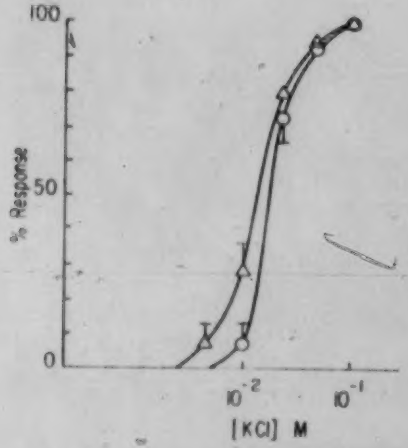


Figure 36. Effect of phenoxybenzamine (2 x 10^{-5}M for 3 min) on response to potassium chloride. Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 4. Bars represent standard errors.

o—o Control dose-response curve

Δ—Δ Dose-response curve after phenoxybenzamine.

phenoxybenzamine shown in Fig. 35 differes somewhat from the type of blockade observed by Cook (1971) in which immediate depression of the maximum response was observed. This difference is most likely due to the fact that, unlike the earlier report, thiosulphate ion was used in the experiments reported in this thesis. This ion removes the aziridinium ion of phenoxybenzamine (see Chapter II - Section C) and thus relieves a reversible component of the blockade due to this species. The mechanism by which this occurs is described in detail in a later section.

The selectivity at the drug receptor level is still evident as the responses to potassium chloride (Fig. 36) and barium chloride (Fig. 37) were not significantly different from controls after administration of phenoxybenzamine.

C. DOSE DEPENDENCY OF BLOCKADE BY PHENOXYBENZAMINE

In the absence of thiosulphate ion, small concentrations of phenoxybenzamine caused depression of the maximum response to histamine. The maximum parallel shift with retention of maximum response that could be achieved under these conditions was 0.67 ± 0.07 log units (Fig. 38). This result is similar to that obtained previously (Cook, 1971) and suggests that the maximum parallel shift without depression of maximum response, under these conditions is independent of whether the measurements are carried out isotonically, as in this present study, or isometrically as done previously. With thiosulphate (10^{-3}M) in the wash medium, increases in the dose of phenoxybenzamine cause an increase in the shift of the dose-response curve to histamine as can be seen in Fig. 39. The maximum shift observed

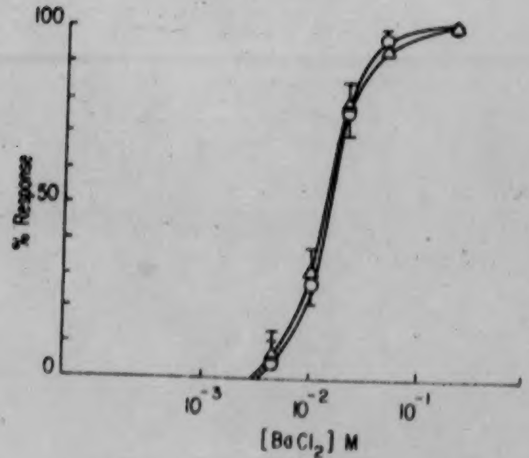


Figure 37. Effect of phenoxybenzamine (2 x 10^{-3}M for 3 min) on response to barium chloride. Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 4. Bars represent standard errors.

o—o control dose-response curve
Δ—Δ dose-response curve after phenoxybenzamine

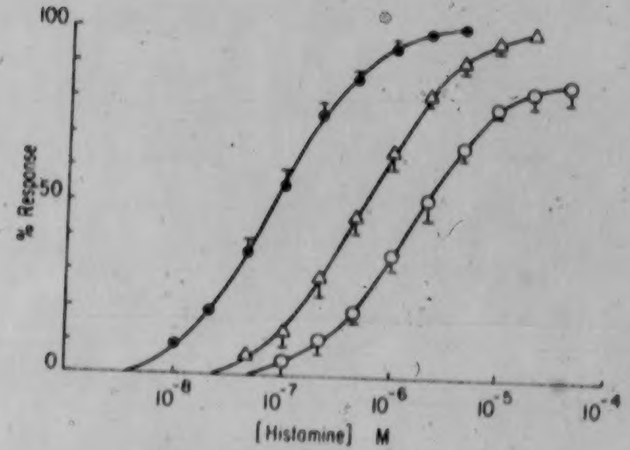


Figure 38. Dose response curves for histamine. Thiosulphate ion not present during wash. Molar concentrations as abscissa, percentage of maximum response as ordinate. Bars represent standard errors.

●—● control response to histamine
Δ—Δ after phenoxybenzamine (7 x 10^{-7}M for 5 min) washed for 2 hours (N=16)
o—o after phenoxybenzamine (10^{-6}M for 3 min), washed for 2 hours (N = 16)

before depression of the maximum response was 1.97 ± .03 log units (5×10^{-6}M for 3 min). This result is the mean of values from 2 separate samples of tissue blocked with phenoxybenzamine that did not produce signifigantly different maximum shifts of the dose-response curves. Higher concentrations of phenoxybenzamine or longer exposure times produced a depression of the maximum response. The slopes of the shifted curves are not signifigantly different at the ED50 except for the maximally shifted dose-response curve (5×10^{-6}M for 3 min). The slope of this curve is signifigantly greater than control at the ED50; the slopes are shown in Table 1. Figure 40 shows the plot of the probit lines for the first four dose-response curves shown in Fig. 39. The slopes of these probit lines are not signifigantly different (Student's t-test) indicating the parallel nature of the shift. The probits for the curves shifted by phenoxybenzamine (5×10^{-7}M for 3 min-Fig. 47),(5×10^{-6}M, 3 min-Fig. 42), however, are signifigantly greater than controls. The slopes of these probit lines are shown in Table 2 but must be considered with caution since the dose-response curves from which they were derived contained fewer points than is necessary for accurate estimates of the slopes by probit transformations. These experiments were carried out to obtain an accurate measure of the ED50 and maximum response and only a few points were required for this purpose.

D. BLOCKADE OF HISTAMINE RESPONSE BY SY14

The maximum irreversible β-haloalkylamine-induced shift of the dose-response curve with retention of the maximum response is, in terms of the spare receptor hypothesis, determined by the magnitude of the receptor reserve. It was thus of interest to observe the

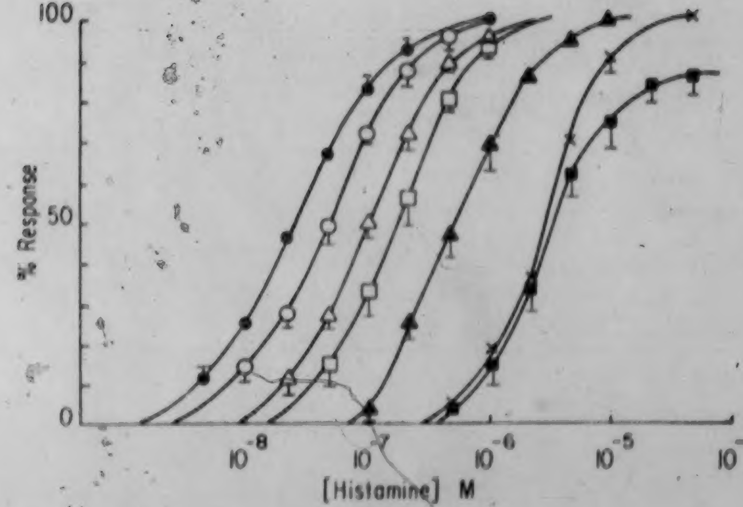


Figure 39. Shift of the dose-response curve to histamine as a result of blockade by phenoxybenzamine dose-response curve to histamine. Molar concentration as abscissa, percent maximum response as ordinate. Bars represent standard errors.

●—● control dose-response curve for histamine (N = 42, pooled controls)
o—o response after phenoxybenzamine (2 x 10^{-8}M for 3 min) (N = 4)
△—△ response after phenoxybenzamine (5 x 10^{-8}M for 3 min) (N = 7)
□—□ response after phenoxybenzamine (2 x 10^{-7}M for 3 min) (N = 5)
▲—▲ response after phenoxybenzamine (5 x 10^{-7}M for 3 min) (N = 7)
x—x response after phenoxybenzamine (5 x 10^{-6}M for 3 min) (N = 14)
■—■ response after phenoxybenzamine (10^{-5}M for 3 min) (N = 5)

TABLE I

Slopes and Shifts of Dose-Response Curves for Tissues Treated with Phenoxybenzamine

Number of Tissues	Slope of Control Curve at ED50	Concentration of Phenoxybenzamine	Slope of Blocked Curve	Shift in Log Units
4	6.90 ± 3.40	2×10^{-8}M for 3 min	8.00 ± 1.56	0.46 ± 0.05
7	6.60 ± 0.40	5×10^{-8}M for 3 min	8.00 ± 1.00	0.79 ± 0.05
5	8.33 ± 0.90	2×10^{-7}M for 3 min	9.40 ± 1.15	1.15 ± 0.07
7	7.76 ± 0.89	5×10^{-7}M for 3 min	7.81 ± 0.77	1.46 ± 0.04
14	5.50 ± 0.40	5×10^{-6}M for 3 min	7.40 ± 0.40*	1.97 ± 0.03

*significantly different from control ($p < 0.01$)

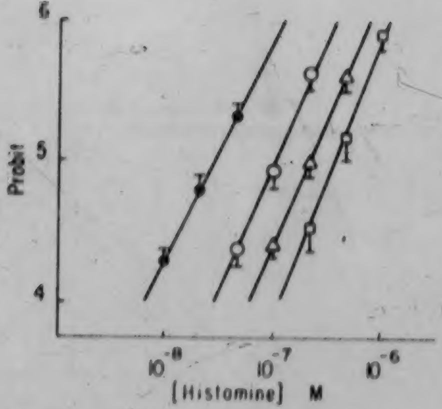


Figure 40. Probits for dose-response curves to histamine after treatment with phenoxybenzamine. Molar concentration as abscissa, probit of percent maximum as ordinate. Bars represent standard errors.

●—● probit for control dose-response curves

o—o probit for dose-response curve to histamine after phenoxybenzamine (2×10^{-8}M for 3 min) (N = 4)

△—△ probit for dose-response curve to histamine after phenoxybenzamine (5×10^{-8}M for 3 min) (N = 7)

□—□ probit for dose-response curve to histamine after phenoxybenzamine (2×10^{-7}M for 3 min) (N = 5)

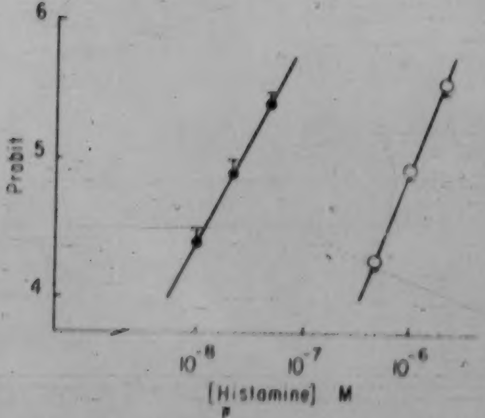


Figure 41. Probit for dose-response curves to histamine after treatment with phenoxybenzamine. Molar concentration as abscissa, probit of percent maximum as ordinate. Bars represent standard errors.

●—● probit for control dose-response curve

o—o probit for dose-response curve to histamine after phenoxybenzamine (5×10^{-7}M for 3 min) (N = 7)

TABLE II

Slopes of Probit Lines for Dose-Response Curves Shifted by Phenoxybenzamine

Number of Tissues	Slope of Probit for Controls	Concentration of Phenoxybenzamine	Slope of Probit after Phenoxybenzamine
4	1.60 ± 0.13	2 x 10^{-8}M for 3 min	1.56 ± 0.24
7	1.71 ± 0.15	5 x 10^{-8}M for 3 min	1.78 ± 0.21
5	1.78 ± 0.10	2 x 10^{-7}M for 3 min	1.92 ± 0.17
7	1.60 ± 0.12	5 x 10^{-7}M for 3 min	1.82 ± 0.12[1]
14	1.39 ± 0.09	5 x 10^{-6}M for 3 min	1.75 ± 0.1[2]

[1] Slopes are significantly different from control ($p < 0.05$).

[2] Slopes are significantly different from control ($p < 0.01$).

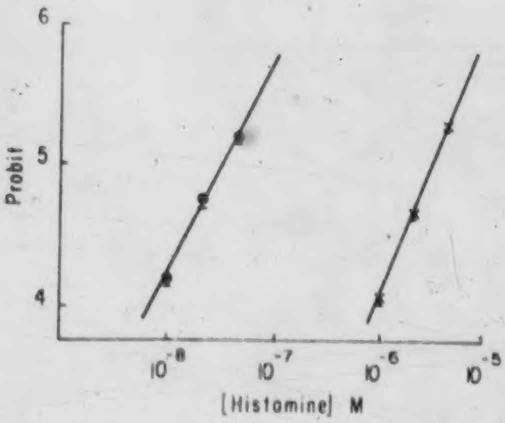


Figure 42. Probit for dose-response curves to histamine after treatment with phenoxybenzamine. Molar concentrations as abscissa, probit of percent maximum as ordinate. Bars represent standard errors.

●—● probit for control dose-response curves
x—x probit for dose-response curve to histamine after phenoxybenzamine (5 x 10^{-6}M for 3 min) (N = 14)

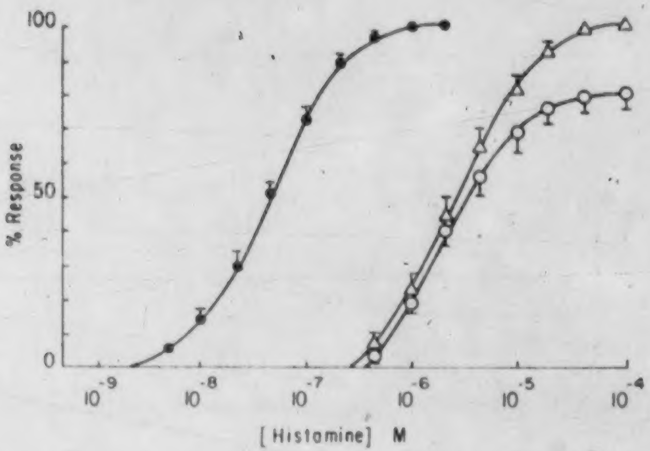


Figure 43. Maximum SY14-induced shift of the dose-response curve. Dose-response curves for histamine. Molar concentrations as abscissa, percentage of maximum response as ordinate. Bars represent standard errors.

●—● control response to histamine
△—△ after 2 x 10^{-7}M SY14 (exposure time of 3 min). Washed with Tyrode solution containing sodium thiosulphate for 3 hours. (N = 8).
o—o after 5 x 10^{-7}M SY14 (exposure time of 3 min). Washed as above. (N = 4).

maximum shift of the dose-response curve, induce by other β-haloalkylamines, and to compare this to the data for phenoxybenzamine.

Treatment of longitudinal smooth muscle strips from guinea pig ileum with SY14 (2×10^{-7}M for 3 min) produced a shift of 1.89 ± .05 log units, in the dose-response curves to histamine. The magnitude of the SY14-induced shift of the dose-response curve was signifigantly different ($p < 0.05$) from that produced by phenoxybenzamine (a sample of 11 tissues blocked by phenoxybenzamine was compared to a sample of 8 tissues blocked by SY14 by a Student's t-test). At higher concentrations, SY14 (5×10^{-7}M, 3 min) produced a depression of the maximum response to histamine (Fig. 43); the time course of the recovery and therefore the reversibility of this depression was not determined.

Aqueous hydrolysis of the aziridinium ion of β-haloalkylamines yields the corresponding alcohol. The alcohol of SY14 was a weak antagonist of the response to histamine (Fig. 44). The antagonism by this agent was readily removed by wash and appears to be of the competitive reversible type. Because high concentrations of SY14 alcohol is required for blockade of the histamine responses, relative to the concetnration of SY14 itself, and because antagonism by the alcohol is readily reversed by wash, unlike SY14, it is unlikely that this hydrolysis product plays a signifigant role in the irreversible blockade of the histamine response by SY14.

E. BLOCKADE OF THE HISTAMINE RESPONSE BY N-ETHYL-N-(2-CHLOROETHYL)-BENZYLAMINE (ECB)

As shown in Fig. 45, ECB (2×10^{-5}M, 3 min) produced a shift of the dose-response curve to histamine of 1.53 ± .03 log units

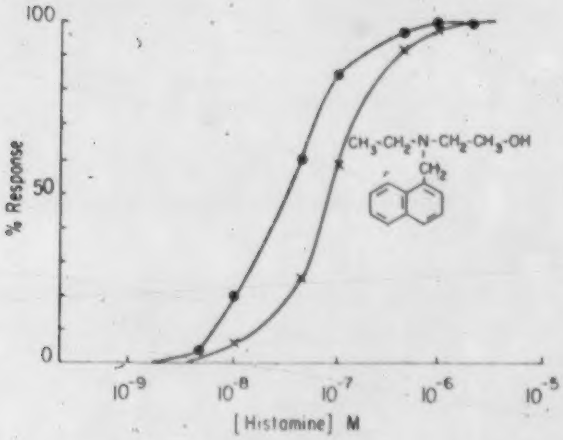


Figure 44. Effect of SY14 alcohol on response to histamine. Dose-response curve for histamine. Molar concentration as abscissa, percent maximum response as ordinate. N = 1.

●—● control dose-response curve

x—x dose-response curve in the presence of alcohol of SY14 (4 x 10^{-5}M)

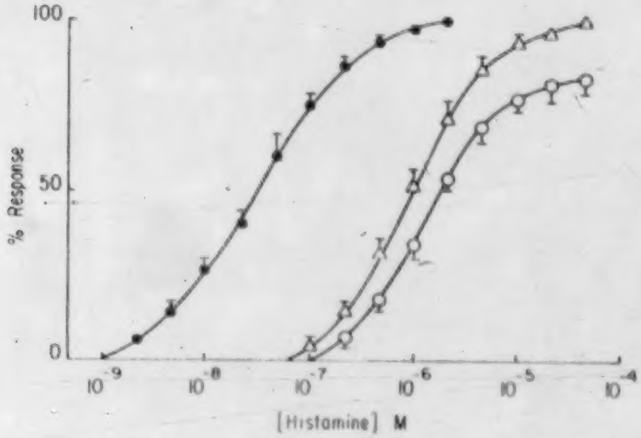


Figure 45. Dose response curves for histamine. Molar concentrations as abscissa, percentage of maximum response as ordinate. Bars represent standard errors.

●—● control response to histamine

Δ—Δ after 2 x 10^{-5}M ECP (exposure time of 3 min). Washed for 3 hours with Tyrode solution containing 1 mM thiosulphate. (N = 10).

o—o after 4 x 10^{-5}M ECP (exposure time of 5 min). Washed as above. (N = 11).

Higher concentrations of this agent or longer exposure times did not increase this shift without depressing the maximum response (Fig. 45) thus the maximum parallel shift with retention of the maximum to histamine caused by FCB is significantly different ($p < .05$) from that produced by either phenoxybenzamine (a separate phenoxybenzamine control from that for SY14) or SY14 (see Table 3).

F. RECEPTOR RESERVES CALCULATED FOR PHENOXYBENZAMINE, SY14, AND FCB

The double reciprocal calculation described in Methods - Section F utilizing the magnitude of the maximum parallel shift in the dose response curve with retention of the maximum response was used to provide values for the apparent receptor reserve for histamine in our preparation. As the receptor reserve is a unique property of the preparation, measurements of this quantity, with different irreversible agents, should yield equivalent results. The double reciprocal plot shown in Fig. 46 and the subsequent calculations (Table 3), however, indicate that the receptor reserve as measured by FCB is significantly smaller than that measured by either phenoxybenzamine ($p < .05$) or SY14 ($p < .05$).

It is theoretically possible to calculate a value for "q", the fraction of receptors irreversibly inactivated (see Chapter II - Section F) from the magnitude of the shift induced in the dose response curve. This calculation, however, would require the assumption that the shift in the dose response curve was parallel, an assumption which cannot be made in light of the results reported in Section C of this chapter. Equiactive doses before and after blockade were therefore compared for each set of dose response curves and an estimate

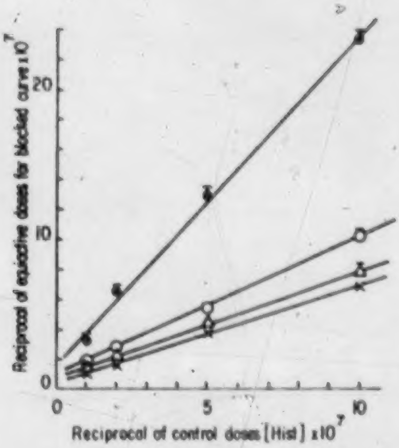


Figure 46. Double reciprocal plots for calculation of "q" - the fraction of receptors irreversibly blocked by various β-haloalkylamines. Reciprocal doses for control curve as as abscissa, reciprocal of equiactive doses for blocked curve as ordinate.

●—● EPC (2 x 10^{-5}M for 3 min, N = 8)
o—o SY14 (2 x 10^{-7}M for 3 min, N = 8)
Δ—Δ phenoxybenzamine (5 x 10^{-6}M for 3 min, N = 8) sample POB-E (see Table III)
x—x phenoxybenzamine (5 x 10^{-6}M for 3 min, N 11) sample POB-S (see Table III)

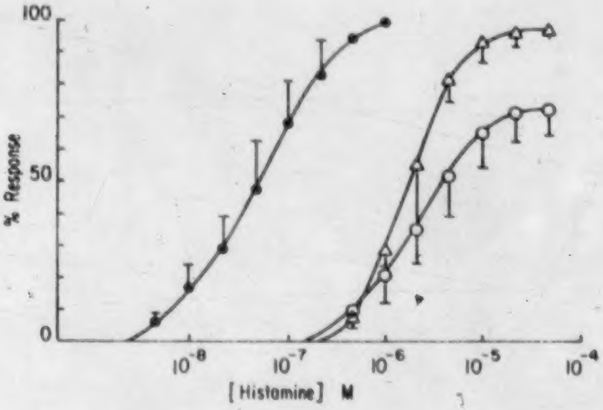


Figure 47. Effect of thiosulphate ion on phenoxybenzamine-induced depression of maximum response to histamine. Dose response curve for histamine. Molar concentration as abscissa, percent maximum response as ordinate. N = 5. Bars represent standard errors.

●—● control dose-response curve
o—o dose-response curve ofter phenoxybenzamine (5 x 10^{-6}M for 3 min and 3 hour wash with normal Tyrode solution
Δ—Δ same tissues after 1 hour wash with Tyrode solution containing 1 mM thiosulphate

TABLE III

Estimates of Apparent Receptor Reserves for Histamine

	POB-E	ECP	POB-S	SY14
Shift at the ED50 (log units)	1.99 ± 0.03 n = 8	1.53 ± 0.03[1] n = 8	1.96 ± 0.01 n = 11	1.89 ± 0.05[2] n = 8
Receptor Reserve	99.18 ± 0.07 n = 8	97.85 ± 0.46[3] n = 8	99.32 ± 0.13 n = 11	98.98 ± 0.12[4] n = 8
Critical Fraction for Maximum Response	0.82 ± 0.05	2.15 ± 0.37	0.68 ± 0.17	1.02 ± 0.26

[1] Curve shift at ED50 significantly different from that for POB-E ($p < 0.01$).

[2] Curve shift at ED50 significantly different from that for POB-S ($p < 0.05$).

[3] Receptor reserve significantly different from that for POB-E ($p < 0.01$).

[4] Receptor reserve significantly different from that for ECP ($p < 0.05$).

of the receptor reserve of each tissue obtained in this manner.

G. REVERSAL OF BLOCKADE BY PHENOXYBENZAMINE WITH THIOSULPHATE ION

It was important to determine whether any part of the blockade produced by these alkylating agents was reversible. Any readily reversible phase of antagonism would imply that a portion of the blockade was due to a reversible complex between antagonist and receptor and not a covalently-bound species. It was found that the maximum β-haloalkylamine induced shift of the dose-response curve was not reversed by wash procedures designed to remove aziridinium ion from the bathing medium. The depression of maximum response, however, was reversed by these procedures.

After tissues were exposed to phenoxybenzamine (5 x 10^{-6}M for 3 min) and washed with Tyrode solution for 3 hours, the maximum response to histamine was 72 ± 8.1% of control. When sodium thiosulphate (10^{-3}M) was then added to the wash medium and washing continued for a further 30 to 60 minutes, a sizeable portion of the blockade was reversed and the maximum response of the tissue was 97 ± 4.2% of control (Fig. 47). As can be seen from Fig. 48, thiosulphate ion itself had no effect on the response to histamine. The reversal of blockade could be greatly enhanced by the presence of 0.5% BSA in medium already containing thiosulphate (Fig. 49). The time course of the reversal of the phenoxybenzamine induced depression of the maximum response can be seen in Fig. 50 (least squares fit). Although the blockade can be reversed by washing with normal Tyrode solution, the $t_{\frac{1}{2}}$ for this process is very long ($t_{\frac{1}{2}}$ = 1150 min). With BSA and thiosulphate ion in the solution, the process was clearly faster ($t_{\frac{1}{2}}$ = 200 min). Although

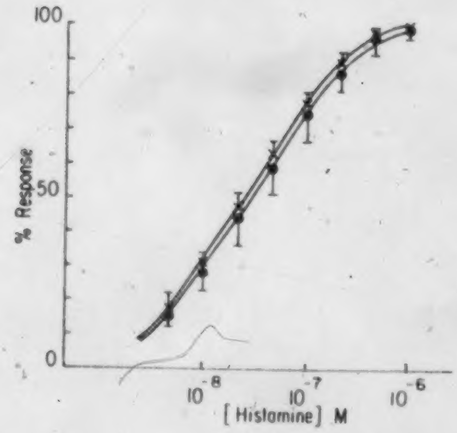


Figure 48. Effect of thiosulphate ion on response to histamine in guinea pig ileum longitudinal muscle. Dose-response curve for histamine. Molar concentrations as abscissa, percent maximum response as ordinate. N = 3. Bars represent standard errors.

●——● control response for histamine

x——x response to histamine after 6 hours wash with Tyrode solution containing thiosulphate ion ($10^{-3}M$)

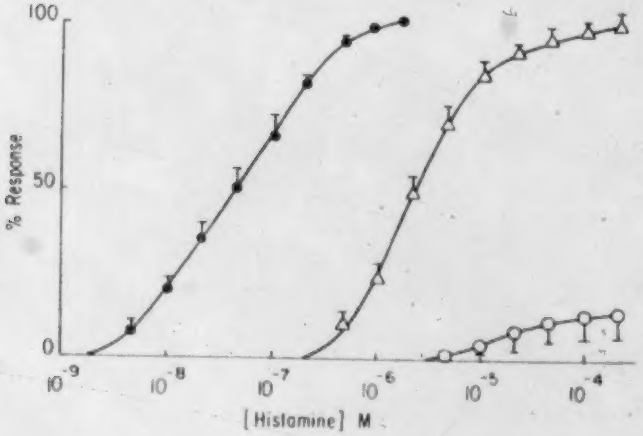


Figure 49. Dose response curves for histamine. Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 6. Bars represent standard errors.

●——● control response for histamine

o——o after 5 x 10^{-5}M phenoxybenzamine (exposure time of 3 min) thiosulphate or BSA not present in wash. Washed for 4 hours.

Δ——Δ same tissues after 2 hours wash with Tyrode solution containing 1 mM thiosulphate/0.5% BSA

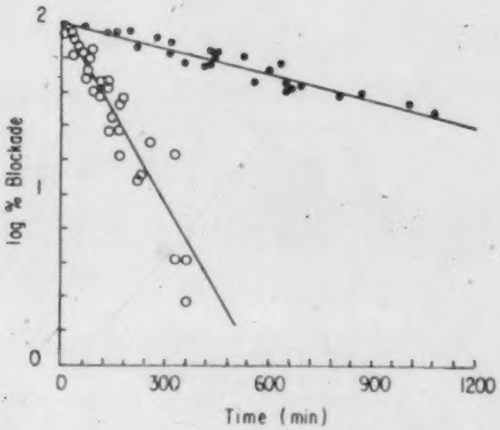


Figure 50. Reversal of phenoxybenzamine blockade with time. Time in minutes as abscissa, log % blockade of maximum response as ordinate.

●—●	tissues washed with normal Tyrode solution, $t_{1/2}$ = 1160 min (N - 8)
o—o	tissues washed with Tyrode solution containing 1 mM thiosulphate/0.5% BSA, $t_{1/2}$ = 200 min (N = 8)

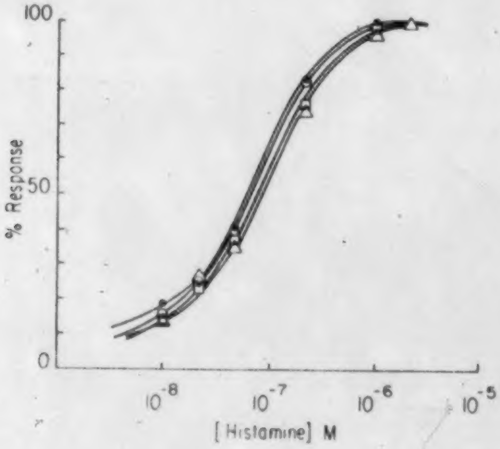


Figure 51 Effect of mercaptopyruvate on response to histamine. Molar concentrations as abscissa, percent maximum response as ordinate. N = 2. Bars represent standard errors.

●—●	control dose-response curve
o—o	dose-response curve after loading with mercaptopyruvate (10^{-3}M for 30 min)
Δ—Δ	dose-response curve after 2 hour wash
▫—▫	dose-response curve after 4 hour wash

thiosulphate ion was capable of reversing 20 to 30 percent of the depression of maximum response caused by phenoxybenzamine, it was incapable of causing further reversal. It appears that some portion of the aziridinium ion is inaccessible to the thiosulphate since BSA was required to provide maximum reversal of blockade. These results suggest that the observed depression of maximum response was not the result of an alkylated drug-receptor complex but rather of a tight binding of the aziridinium ion of phenoxybenzamine. It is important to point out that under no circumstances could thiosulphate ion or BSA reverse the shift of the dose-response curve thus implying that measurable differences in the shift of the dose-response curve are smaller than experimental error (correlation coefficient = 0.22). The presence of thiosulphate ion and BSA in the wash did not alter the behavior of a shifted curve with respect to the wash time - there was no detectable difference in the calculated fractions of alkylated receptors at various wash times. This suggests that the phenoxybenzamine-induced shift of the dose-response curve is due to a covalently bonded complex.

H. REVERSAL OF THE PHENOXYBENZAMINE-INDUCED DEPRESSION OF MAXIMUM RESPONSE WITH MERCAPTOPYRUVATE

In view of the recent reports indicating that phenoxybenzamine is able to enter into the cell cytoplasm and nucleus (Graham _et_ _al_., 1968; Graham _et_ _al_., 1971; Mottram, 1974), it was of interest to try to determine whether a component of the blockade of the response to histamine, induced by phenoxybenzamine, was due to intracellular alkylation of components of the excitation-contraction mechanism.

Sorbo (1957a) reported that the agent mercaptopyruvate is capable of entering cells and taking part in a mechanism which produces intracellular thiosulphate ion. This thiosulphate ion could then inactivate any intracellular phenoxybenzamine. Sorbo (1957a) also stated that mercaptopyruvate itself is capable of reacting with aziridinium ion.

If a portion of the blockade is due to alkylation of an intracellular component of the mechanism responsible for the response to histamine, then presumably pretreatment of a tissue with mercaptopyruvate would generate an intracellular concentration of species capable of scavenging aziridinium ion and thus protect against any intracellular component of blockade. Fästh and Sorbo (1973) found that pretreatment of cells for 30 minutes with mercaptopyruvate led to a maximum intracellular level of thiosulphate. The maximum dose of mercaptopyruvate which had no noticeable effects on the response to histamine when the tissue was exposed to it for 30 min was 10^{-3}M (see Fig. 51). However, no detectable amount of thiosulphate ion was observed in tissues pretreated with mercaptopyruvate in this manner. Pretreatment of tissues with mercaptopyruvate had no effect on the blockade of histamine response produced by phenoxybenzamine. Mercaptopyruvate, however, did affect the rate of reversal of the phenoxybenzamine-induced depression of the maximum response. Figure 52 shows the sizeable increase in the rate of recovery from blockade by phenoxybenzamine in tissues pretreated for 30 minutes with mercaptopyruvate (10^{-3}M). The $t_{\frac{1}{2}}$ for the reversal of the depression of the maximum response was 280 minutes as compared to a $t_{\frac{1}{2}}$ of 1150 minutes for control tissues (least squares fit). No reversal of the

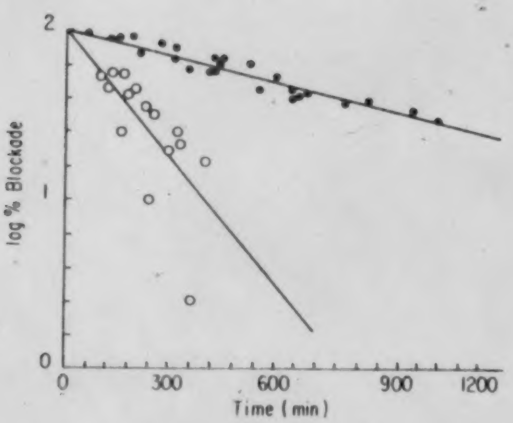


Figure 52. Reversal of phenoxybenzamine blockade with time. Time in minutes as abscissa, log % blockade of maximum response as ordinate.

●—● tissues washed with normal Tyrode solution $t_{½}$ = 1150 min (N = 8)

o—o tissues washed with Tyrode solution after 30 min loading with 10^{-3}M mercaptopyruvate, $t_{½}$ = 280 min (N = 4)

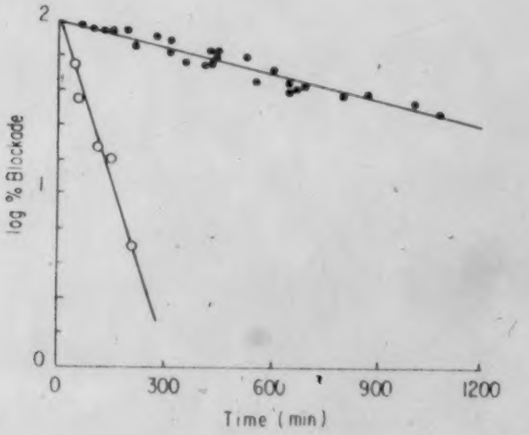


Figure 53. Reversal of phenoxybenzamine blockade with time. Time in minutes as abscissa, log % blockade of maximum response as ordinate.

●—● tissues washed with normal Tyrode solution $t_{½}$ = 1150 min (N = 8)

o—o tissue washed with Tyrode solution containing 0.5% BSA/10^{-3}M thiosulphate ion after loading with mercaptopyruvate (10^{-3}M for 30 min) $t_{½}$ = 100 min (N = 1) phenoxybenzamine-induced shift of the dose-response curve was observed after pretreatment of the tissues with mercaptopyruvate.

The effects of mercaptopyruvate and BSA/thiosulphate appea to be additive as the $t_{\frac{1}{2}}$ for recovery, when both treatments were used, was less than that for either treatment alone ($t_{\frac{1}{2}} \approx 100$ min, Fig. 53).

I. TIME COURSE OF AZIRIDINIUM ION PRODUCTION AND DECAY

Since the depression of maximum response by phenoxybenzam appears to be due to the aziridinium ion of this antagonist, this must be shown to be chemically stable during this time period. F 54 shows the production and decay with time of the aziridinium io of phenoxybenzamine in aqueous solution. The values on the ordina are percentages of the maximum amount of aziridinium ion detected any one experiment. The concentration of aziridinium ion is propo tional to the amount of red dipicrylamine anion formed in the assa medium which is in turn measured by the amount of absorbance at 4 As there is no standard concentration of aziridinium ion with whi to compare the values of absorbance, they cannot be readily conve into molar quantities. The percentages were obtained by comparin the absorbances at the arbitrary assay times and therefore the ac peak of the concentration of the aziridinium ion may or may not h been observed by this method.

Since Fig. 54 is the mean of seven determinations and as maximum concentration of aziridinium ion was not obtained at the sampling time for each experiment, the ordinate value did not rea 100%. Consistent measurements were difficult to obtain in this r of the graph as the rate of cyclization was very fast in the initial time periods (5 min to 20 min). The primary function of this assay, however, was to ascertain whether or not there was a detectable concentration of aziridinium ion present in the aqueous solution at pH 7.4 and at 37°C. As seen in Fig. 54, there appears to be a significant amount of aziridinium ion present even after 3 hours under these conditions.

J. DMPEA AS AN AGONIST IN GUINEA PIG ILEUM

As DMPEA has been found to be an irreversible α-blocking agent with remarkable properties (Moran, Triggle and Triggle, 1969), attempts were made to study blockade of histamine receptors by this agent. DMPEA was found to be a full agonist in this preparation (Fig. 55). When DMPEA was preincubated with thiosulphate ion, the agonist properties were lost, indicating that the species responsible was most likely the aziridinium ion of DMPEA (Fig. 56).

It has been suggested that DMPEA has "acetylcholine-like" activity (Graham and James, 1961). Responses to DMPEA were not blocked by concentrations of SY14 sufficient to significantly diminish contractions to histamine. Antagonism of contractions in response to DMPEA was observed, however, when higher concentrations of SY14, capable of blocking responses to acetylcholine, were used (Fig. 57). Estimates of pA_2 values for atropine, using acetylcholine and DMPEA, were not greatly different (Table IV) providing further evidence for the muscarinic effects of this species.

The contractile response to DMPEA was also tested on ileum which had been isolated with an intact nerve supply and mesentery.

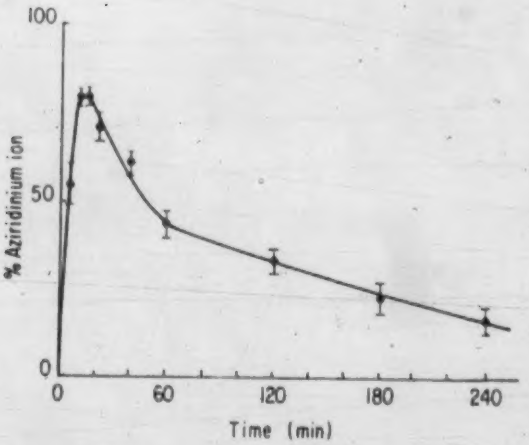


Figure 54. Production and decay with time of aziridinium ion for phenoxybenzamine, in aqueous buffer. Percent formation of aziridinium ion as ordinate (see Chapter II - Section E), time in minutes as abscissa. N = 7. Bars represent standard errors.

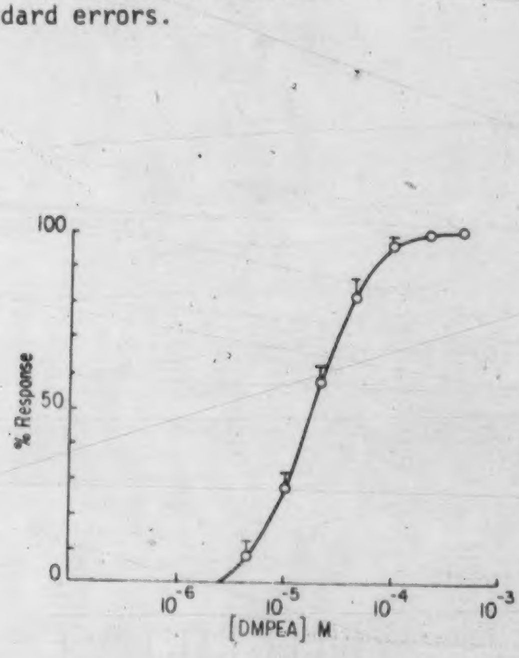


Figure 55. Dose-response curve of guinea pig ileum to N,N-dimethyl-1-bromophenylethylamine (DMPEA). Molar concentration DMPEA as abscissa, percent maximum response as ordinate. N = 6. Bars represent standard errors.

TABLE IV

pA_x Values Against Agonists for Various Antagonists

Antagonist	Agonist	pA_2	pA_5
Diphenhydramine	Histamine	11.11	9.11
Diphenhydramine	DMPEA	10.91	9.41
Atropine	Acetylcholine	8.28 *	
Atropine	DMPEA	8.11 *	
DMPEA alcohol	Histamine	7.51	
DMPEA alcohol	Acetylcholine	6.51	
DMPEA alcohol	DMPEA	6.50	

* These values are lower than those found in the literature. They were obtained after Atropine had been equilibrated with the tissue for only 10 minutes. This short equilibration time could account for the discrepancy.

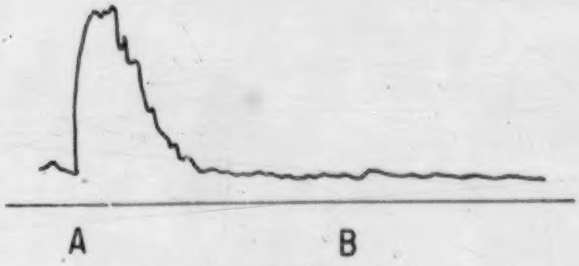


Figure 56. Isotonic contractions of guinea pig ileum to DMPEA.

A - DMPEA (3 x 10^{-5}M)

B - DMPEA (3 x 10^{-5}M) preincubated with thiosulphate ion (10^{-3}M) for 10 minutes

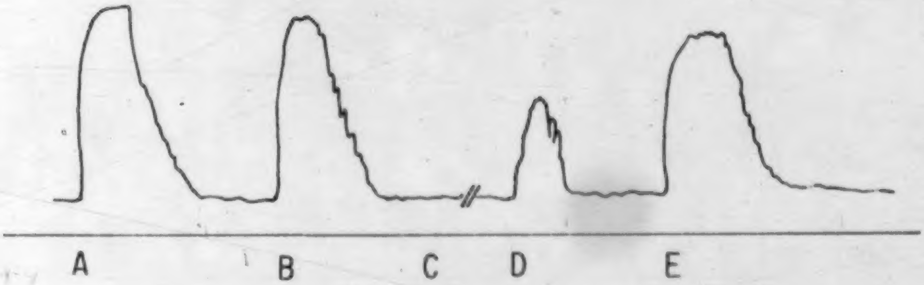


Figure 57. Isotonic contractions of guinea pig ileum to DMPEA and histamine.

A - response to histamine (10^{-6}M)

B - response to DMPEA (3 x 10^{-5}M)

C - SY14 (5 x 10^{-6}M for 5 min)

D - response to histamine (10^{-4}M)

E - response to DMPEA (10^{-4}M)

The nerve was stimulated with bipolar electrodes (60 Hz) and contractions, caused by release of neuronal acetylcholine, recorded isotonically (Finkelman, 1930). The preparation was stimulated for 2 hours in the presence of hemicholinium (10^{-5}M), an agent thought to prevent uptake of choline necessary for biosynthesis of acetylcholine, into the nerve terminals (Birks and MacIntosh, 1961). After the 2 hours of stimulation, the neuronal acetylcholine stores were depleted to the extent that nerve stimulation caused minimal contraction, while response to exogenously administered acetylcholine was unaffected. Under these circumstances, in a single experiment, the response to DMPEA was reduced to 80% of control. These results suggest that DMPEA may possess a small indirect component of action resulting from the release of neuronal acetylcholine.

K. DMPEA AS AN ANTAGONIST OF HISTAMINE

Although DMPEA is known to inhibit histaminase (Graham and James, 1961), at concentrations less than 10^{-3}M it does not significantly antagonize the response to histamine in longitudinal smooth muscle (Fig. 58). In higher doses (10^{-3}M) it produces a small shift of the dose-response curve for histamine which reverses with time (Fig. 58).

The alcohol of DMPEA antagonizes the response to histamine in a reversible manner (Fig. 59). It is not clear, at present, what causes the transient blockade of histamine by DMPEA (10^{-3}M). Whether it is an effect of the alcohol of DMPEA, a brief alkylation (with fast hydrolysis) or a binding of aziridinium ion has not been determined.

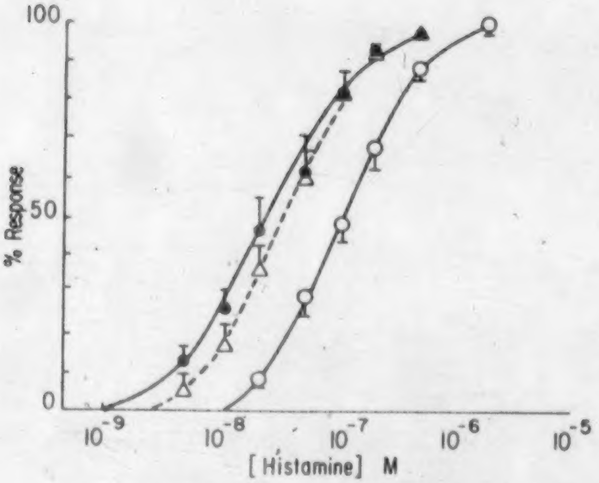


Figure 58. Dose-response curve to histamine. Molar concentration histamine as abscissa percent maximum response as ordinate. Bars represent standard errors.

•—• control response to histamine
o—o after DMPEA (10^{-3}M for 5 min), washed for 30 min (N = 7)
Δ---Δ after DMPEA (10^{-5}M for 5 min), washed for 30 min (N = 10)

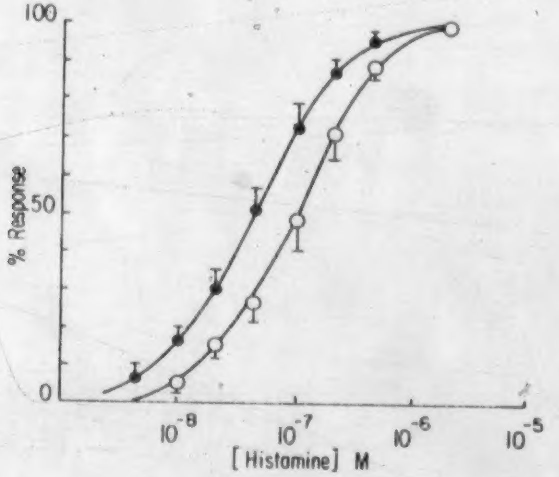


Figure 59. Effect of DMPEA alcohol on response to histamine. Dose-response curve for histamine. Molar concentration as abscissa, percent maximum response as ordinate. N = 2.

•—• control dose-response curve
o—o response to histamine in presence of alcohol of DMPEA (7 x 10^{-8}M)

L. EFFECT OF DMPEA ON BLOCKADE BY PHENOXYBENZAMINE OF THE RESPONSE TO HISTAMINE

It is interesting to note that others (Moran, Swami and Triggle, 1970; Janis and Triggle, 1971) have used DMPEA to distinguish more than one binding site for β-haloalkylamines in the α-receptor system. There it was found that DMPEA pretreatment antagonized binding of other β-haloalkylamines at one of the sites. In view of these results obtained with this agent, the effect of pretreatment with DMPEA on the blockade of histamine response by phenoxybenzamine was determined.

Pretreatment of a tissue with DMPEA (5×10^{-5}M for 5 min) did not affect the shift in the dose-response curve produced by phenoxybenzamine (5×10^{-6}M for 3 min, Fig. 60). Table V shows a comparison of the maximum parallel shifts of the dose-response curves to histamine with retention of maximum response with and without DMPEA pretreatment and it can be seen that there is no significant difference. There is a significant difference, however, in the depression of the maximum response produced by phenoxybenzamine (10^{-5}M for 5 min) with and without pretreatment with DMPEA (Fig. 61). With DMPEA pretreatment, the maximum response after phenoxybenzamine was 96 ± 2.2% while in control tissues (phenoxybenzamine only) the maximum response was 76 ± 6.7%. The difference is statistically significant ($p < 0.05$, see Table V).

M. HISTAMINE DESENSITIZATION

In view of the very high concentrations of agonist required for receptor-protection studies and the high probability of resulting

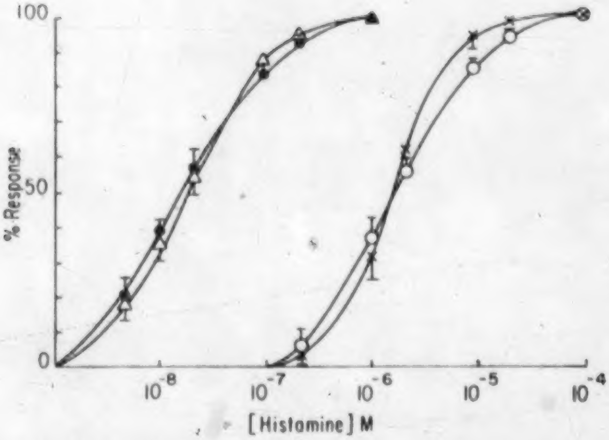


Figure 60. Dose-response curves to histamine. Molar concentration of histamine as abscissa, percent maximum response as ordinate.

●—● control dose-response curve to histamine for tissues pretreated with DMPEA
Δ—Δ control dose-response curve of tissues not pretreated with DMPEA
o—o after DMPEA (5×10^{-6}M for 5 min) and phenoxybenzamine (5×10^{-6}M for 3 min) (N = 4)
x---x after phenoxybenzamine (5×10^{-6}M for 3 min) (N = 21)

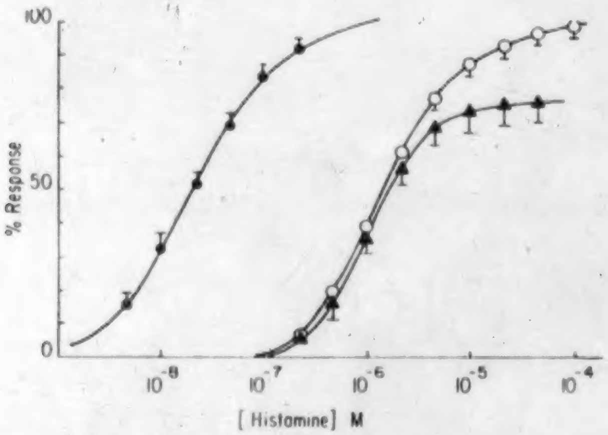


Figure 61. Dose-response curves to histamine. Molar concentration histamine as abscissa, percent maximum response as ordinate. Bars show standard errors.

●—● control response to histamine
o—o after DMPEA (5×10^{-6}M for 5 min) and phenoxybenzamine (10^{-5}M for 5 min) (N = 9)
▲—▲ after phenoxybenzamine (10^{-5}M for 5 min) (N = 5)

All dose-response curves of blocked tissues obtained after a 2 hour wash with Tyrode solution containing 1 mM thiosulphate.

TABLE V

Effect of DMPEA on Phenoxybenzamine-Induced Shift of the Dose-Response Curve and Depression of Maximum Response

	Concentration Phenoxybenzamine	DMPEA Pretreated	Phenoxybenzamine only
Maximum Shift (log units)	5 x 10^{-6}M for 3 min	1.99 ± 0.04 n = 6	1.97 ± 0.03 n = 21
Maximum Response	10^{-5}M for 5 min	96.00 ± 2.2%*	76.00 ± 6.7%

*Maximum response significantly different from that for phenoxybenzamine only ($p < 0.01$).

extensive desensitization, the effect of desensitization on the blockade produced by phenoxybenzamine was determined. Desensitization has already been shown to affect the irreversible blockade of cholinergic receptors (Rang and Ritter, 1970; Miledi and Potter, 1971; Lester, 1972; Dryden and Harvey, 1974) thus it was felt that this effect required investigation in the histamine receptor system.

A non-specific component of histamine desensitization has been characterized (Cantoni and Eastman, 1946; Paton, 1967; Bown et al., 1973; Schild, 1973a) which makes quantitative measurement of ρ , the fraction of histamine receptors desensitized, difficult (see Chapter II - Section H). The effect of desensitization to histamine on the response to acetylcholine was therefore determined in an attempt to measure the extent of the non-specific component of desensitization.

Figure 62 shows the parallel shift induced in a dose-response curve to histamine by a desensitizing dose of histamine. The concomitant desensitization to acetylcholine is shown in Fig. 63, illustrating the non-specific component of the desensitization.

As many of the experiments contained in this thesis suggest that the phenoxybenzamine-induced depression of the maximum response and shift of the dose-response curve result from different mechanisms, it was decided that the effects of desensitization on these two effects should be studied separately.

N. EFFECT OF DESENSITIZATION ON THE PHENOXYBENZAMINE-INDUCED SHIFT OF THE DOSE-RESPONSE CURVE

Paired tissues were utilized to determine differences in the phenoxybenzamine-induced shift of the dose-response curve (in log units)

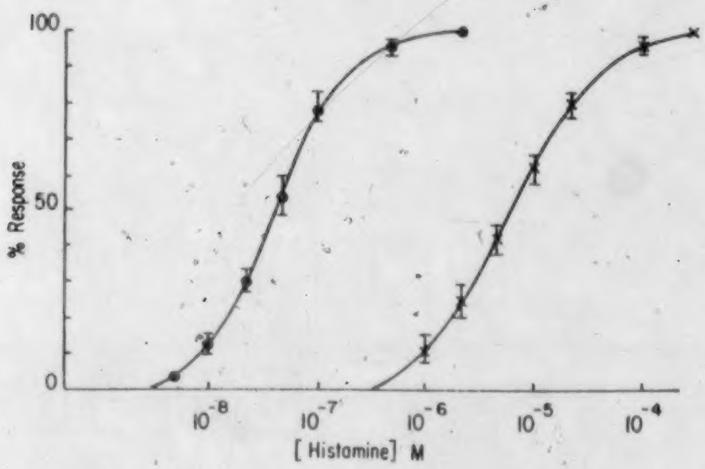


Figure 62. Effect of maximal desensitization to histamine on the response to histamine. Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 20. Bars represent standard errors.

●—● control dose-response curve to histamine

x—x dose-response curve after desensitization to histamine

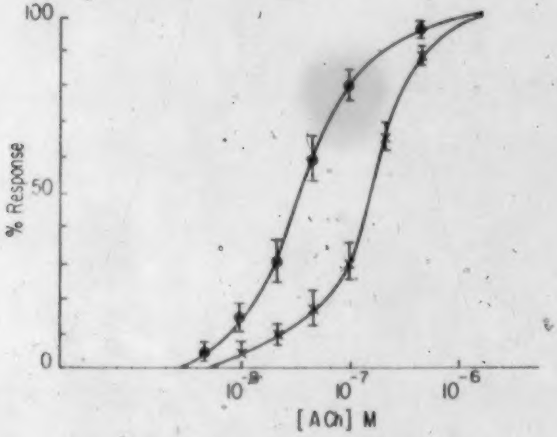


Figure 63. Concomitant desensitization to acetylcholine after desensitization to histamine. Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 6. Bars represent standard errors.

●—● control dose-response curve to acetylcholine

x—x dose-response curve to acetylcholine after desensitization with histamine in desensitized and non-desensitized control tissues. Little difference in the magnitude of the shift of the dose-response curve was observed between desensitized and non-desensitized tissues and there was no correlation (correlation coefficient = 0.26) between differences in the shift and ρ, the measure of desensitization (Fig. 64).

Since in some cases there was a measurable difference in the phenoxybenzamine-induced shift of the dose-response curve between control tissues and tissues which were maximally desensitized ($\rho = 100$), 8 pairs of tissues were utilized in an attempt to determine the significance of this effect. Under these circumstances, a difference of 0.30 ± 0.07 log units was observed (paired t-test, $p < 0.05$; note these 8 maximally desensitized tissues also appear in Fig. 64).

O. EFFECT OF DESENSITIZATION ON THE PHENOXYBENZAMINE-INDUCED DEPRESSION OF THE MAXIMUM RESPONSE

Desensitization had a much more profound effect on the phenoxybenzamine-induced depression of the maximum response to histamine. Figure 65 shows the correlation between ρ, as a measure of desensitization, and Δmax, defined as the difference between the maximum responses of a desensitized and non-desensitized tissue (both blocked by phenoxybenzamine). Desensitization reduces the phenoxybenzamine-induced depression of the maximum response (correlation coefficient = 0.86). Phenoxybenzamine depresses the maximum response of a non-desensitized tissue to 32% while that of a maximally desensitized tissue ($\rho = 100$) is depressed to only 82% compared to unblocked, non-desensitized tissue (Fig. 66).

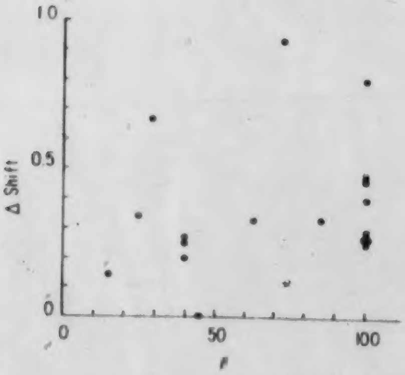


Figure 64. Differences between parallel shift induced in the dose-response curve, by phenoxybenzamine (2×10^{-6}M for 3 min), of desensitized and non-desensitized tissues, against ρ as a measure of desensitization. N = 17. Correlation coefficient = 0.26.

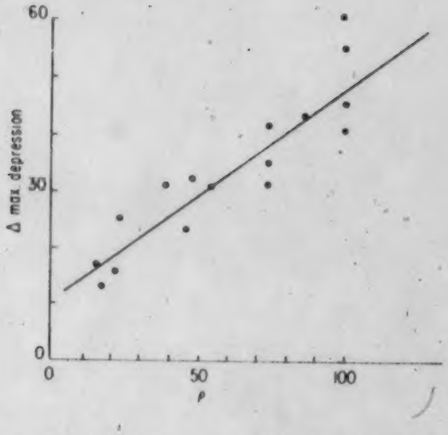


Figure 65. A plot of difference between phenoxybenzamine-induced depression of maximum response to histamine, in desensitized and non-desensitized tissues, and ρ as a measure of desensitization. N = 16. Correlation coefficient = 0.86.

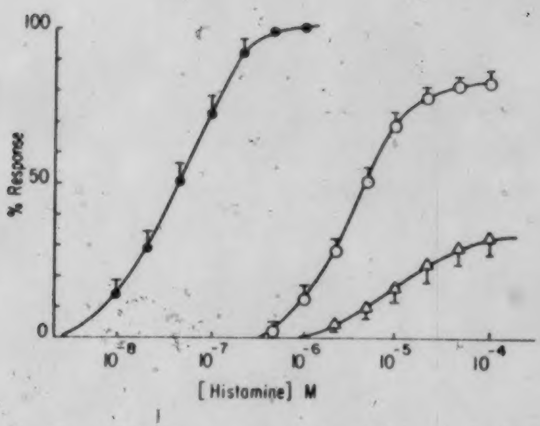


Figure 66. Effect of maximal desensitization (o = 100) on depression of maximum response by phenoxybenzamine (5 x 10^{-6}M for 3 min). Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 4. Bars represent standard errors.

●——● control dose-response curve to histamine
Δ——Δ dose-response curve after phenoxybenzamine
o——o dose-response curve, after phenoxybenzamine, of tissue desensitized before exposure to antagonist

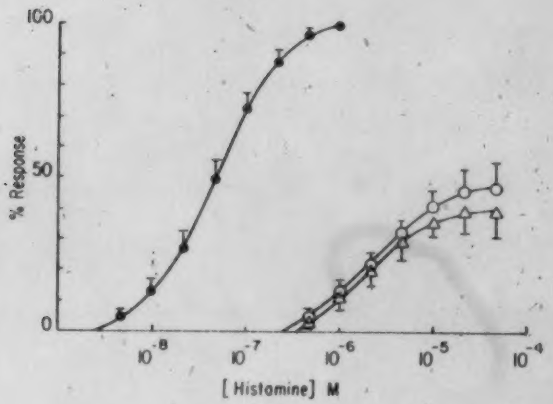


Figure 67. Effect of histamine (10^{-6}M) present in the bath during exposure to phenoxybenzamine (5 x 10^{-6}M for 3 min). Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 6. Bars represent standard errors.

●——● control dose-response curve to histamine
Δ——Δ dose-response curve after phenoxybenzamine
o——o dose-response curve after phenoxybenzamine in presence of histamine (10^{-6}M)

P. RECEPTOR PROTECTION BY A SUBTHRESHOLD CONCENTRATION OF HISTAMINE

One obvious methodological problem is the possible binding-site protecting effect of a subthreshold amount of histamine present in the bath fluid of desensitized tissues. This subthreshold concentration theoretically can be relatively high (up to 10^{-6}M) since a highly desensitized tissue would not respond to such a dose. A second control was therefore included to clarify any possible protecting effect of undetected concentrations of histamine on the antagonism by phenoxybenzamine.

It was found that an agonist concentration of 10^{-6}M produced no effects on the phenoxybenzamine-induced shift of the dose-response curve (see Section S). It was found, however, that this dose of histamine had a small effect on the depression of maximum produced by the β-haloalkylamine. In this case, histamine (10^{-6}M) caused a difference between the depressed maximum response of desensitized and non-desensitized tissues of 8.3 ± 3% (Fig. 67). Such a dose of histamine would produce no response and therefore remain undetected in highly desensitized preparations (ρ = 100) where differences in the depressed maximum response (Δmax) caused by desensitization were found to be considerably greater (50.5 ± 4.6%). Thus the protecting effect of subthreshold doses of histamine does not appear to be a serious complication in the interpretation of the studies concerning desensitization and receptor blockade. It must be remembered that although 10^{-6}M histamine is theoretically the highest concentration which could remain undetected in the tissue bath, there is no evidence to imply that this is the case. If present, however, this residual agonist concentration could account for only a fraction of the observed differences.

It is possible that the histamine molecule remains tightly bound to the receptor for a considerable length of time thereby causing protection against binding of phenoxybenzamine. If there is a receptor reserve of 99% then 10^{-6}M histamine (the lowest concentration which produces maximum response) would only be binding to 1% of the receptor population. To determine whether the effects of desensitization on blockade by phenoxybenzamine was an artifact due to tight-binding of histamine to the desensitized receptor, the effects of a histamine concentration of 10^{-4}M on the blockade by phenoxybenzamine was observed. This concentration of histamine would be sufficient to occupy 100% of the receptors assuming a 99% receptor reserve. The difference between the maximum responses of control tissues and tissues in the presence of 10^{-4}M histamine (Δmax) is 35 ± 7.4% (see Fig. 68). This is considerably smaller than the effect of maximum desensitization on the depression, by phenoxybenzamine, of the maximum response (Δmax = 50.5 ± 4.6%).

Q. INCREASE IN MAGNITUDE OF BLOCKADE BY PHENOXYBENZAMINE WITH TIME

As shown in Fig. 69, there is a significant increase ($p < 0.01$) in the phenoxybenzamine-induced depression of maximum response after 3 hours of wash in normal Tyrode solution not containing thiosulphate ion. These preparations were treated with phenoxybenzamine dissolved in dilute acid thus a larger concentration of uncyclized β-haloalkylmine was present in the bath than is normally found. These results, however, illustrate the ability of this agent to produce blockade long after the initial period of exposure to the tissue. As the

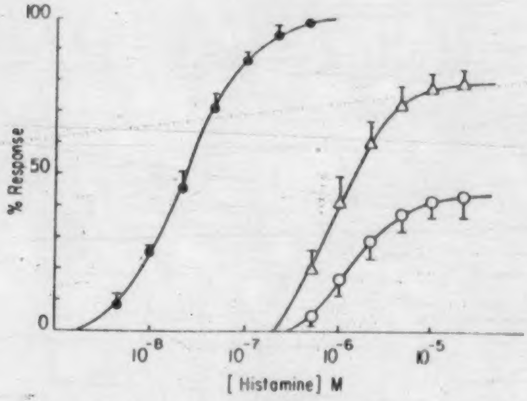


Figure 68. Effect of histamine (10^{-4}M) present in the bath during exposure to phenoxybenzamine (5×10^{-6}M for 3 min). Molar concentrations as abscissa, percentage of maximum response as ordinate. N = 6. Bars represent standard errors.

●—● control dose-response curve to histamine
o—o dose-response curve after phenoxybenzamine
Δ—Δ dose-response curve after phenoxybenzamine in the presence of histamine (10^{-4}M)

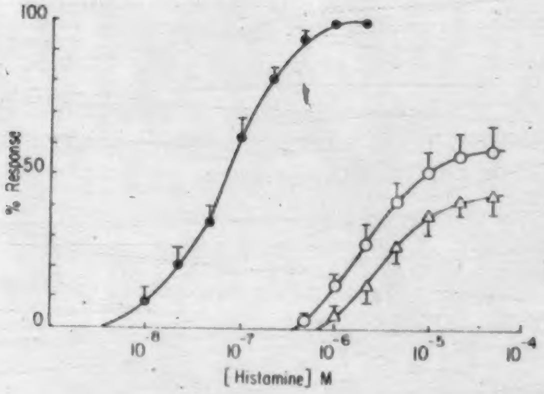


Figure 69. Increase in blockade by phenoxybenzamine with wash in normal Tyrode solution. Dose-response curve for histamine Molar concentration as abscissa, percent maximum response as ordinate. N = 4. Bars represent standard errors.

●—● control dose-response curve
o—o dose-response curve after phenoxybenzamine (5×10^{-6}M for 5 min) and 20 minute wash with normal Tyrode solution
Δ—Δ response in same tissues after 3 hour wash with normal Tyrode solution

effect is not often observed under normal circumstances but does become evident in phenoxybenzamine that is freshly dissolved in acid, it appears that the uncyclized form of phenoxybenzamine is responsible for this continuation of the alkylation process after initial exposure to blocking agent.

R. PHARMACOLOGICAL SIGNIFICANCE OF DOSES OF VARIOUS PROTECTING AGENTS

Much of the work to this point in time indicated that phenoxybenzamine was binding to 2 separate sites associated with the receptor. In an attempt to determine whether or not one of these sites was the histamine binding site, a series of protection studies was initiated. The partial agonist Et_2pyretamine, full agonist histamine and reversible antagonist diphenhydramine were used in various experiments to protect against the blockade of the histamine response by phenoxybenzamine. Many receptor-protection studies suffer from the fact that very large concentrations of protecting agent capable of protecting many non-specific sites are used. Attempts were made to keep the concentrations of protecting agent utilized in the following experiments within a reasonable pharmacological range although the failure of high doses of histamine to affect the phenoxybenzamine-induced shift of the dose-response curve necessitated the use of very high concentrations of this agent (vide infra). The pharmacological significance of the various doses of protecting agent are shown in Table VI.

Although Et_2pyretamine has been described as a partial agonist for histamine (Walter et al., 1941; Hunt and Fosbinder, 1942), no pA_x values are readily available. The pA_2 for diphenhydramine determined against histamine was 7.63 ± 0.02, and against Et_2pyretamine was

TABLE VI

Pharmacological Significance of Various Concentrations of Agents

Agent	Concentration	Significance
Histamine	10^{-6}M	produces maximum response
Histamine	10^{-4}M	100 x dose which produces maximum response
Histamine	10^{-3}M	1000 x dose which produces maximum response
Et_2pyretamine	10^{-5}M	subthreshold dose
Et_2pyretamine	5×10^{-5}M	ED50 dose
Et_2pyretamine	10^{-4}M	produces maximum response
Et_2pyretamine	10^{-3}M	10 x dose which produces maximum response
Diphenhydramine	2×10^{-8}M	occupies 3.25 ± 0.4%* of the receptors
Diphenhydramine	10^{-7}M	occupies 10.9 ± 1.3% of the receptors

*As calculated by equation derived by Paton (1961)

$$\frac{DR-1}{DR} = \rho$$

7.63 ± 0.04. This is close to the value of the pA_2 for diphenhydramine of 7.7 as determined with histamine by Van Rossum (1966b). A dose-response curve for Et_2pyretamine on the longitudinal muscle of the guinea pig ileum is shown in Fig. 70.

In dealing with the pharmacological effects of diphenhydramine, one way to express the extent of receptor occupation by this antagonist is with the equation derived by Paton (1961). The equation (dose ratio - 1)/(dose ratio) (see Chapter II - Section H) can be used to calculate ρ , the fraction of receptors blocked by reversible antagonist. These percentages, for the protecting doses of diphenhydramine, are shown in Table VI.

S. EFFECT OF PROTECTING AGENTS ON SHIFT CAUSED BY PHENOXYBENZAMINE

As shown in the histogram Fig. 71, the effects of supramaximal doses of histamine on the phenoxybenzamine-induced shift in the histamine dose-response curve are minimal. The results of the statistical calculations are shown in Table VII where it can be seen that one hundred times the concentration of histamine required to elicit the maximum response, fails to produce a significant decrease in the shift by phenoxybenzamine. Significant differences in the magnitude of the shift in the dose-response curve were achieved when a high dose of Et_2pyretamine (10^{-3}M) was utilized as the protecting agent. In light of the chemical interactions between Et_2pyretamine and phenoxybenzamine, the full significance of this difference in parallel shift in dose-response curve will be discussed in Section V.

The reversible antagonist diphenhydramine produced a large difference in the phenoxybenzamine-induced shift in dose-response curve.

TABLE VII

Protection by Agonists Against Phenoxybenzamine-Induced Shift of the Dose-Response Curve

Agonist	Concentration	Number of Tissues	ΔShift (log units)	Significant Difference from Control
Histamine	$10^{-6}M$	5	0.05 ± 0.06	not significant
*Histamine	$10^{-6}M$	1	0.01	
Histamine	$10^{-4}M$	6	0.18 ± 0.09	not significant
Histamine	$10^{-3}M$	5	0.11 ± 0.03	$p < 0.05$
Et_2pyretamine	$10^{-5}M$	4	0.08 ± 0.09	not significant
Et_2pyretamine	$10^{-4}M$	5	0.12 ± 0.05	$p < 0.05$
Et_2pyretamine	$10^{-3}M$	4	0.57 ± 0.10	$p < 0.01$
Diphenhydramine	$2 \times 10^{-8}M$	6	0.34 ± 0.13	$p < 0.05$
Diphenhydramine	$10^{-7}M$	4	1.00 ± 0.16	$p < 0.01$

*In the presence of $10^{-4}M$ hydroxylamine

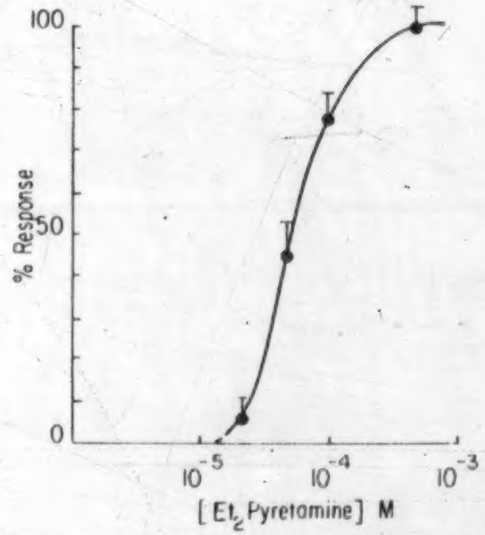


Figure 70. Dose-response curve for Et_2pyretamine. Molar concentration as abscissa, percent maximum response as ordinate (100% refers to maximum for Et_2pyretamine - this is approximately 80% of maximum for histamine). N = 10. Bars represent standard errors.

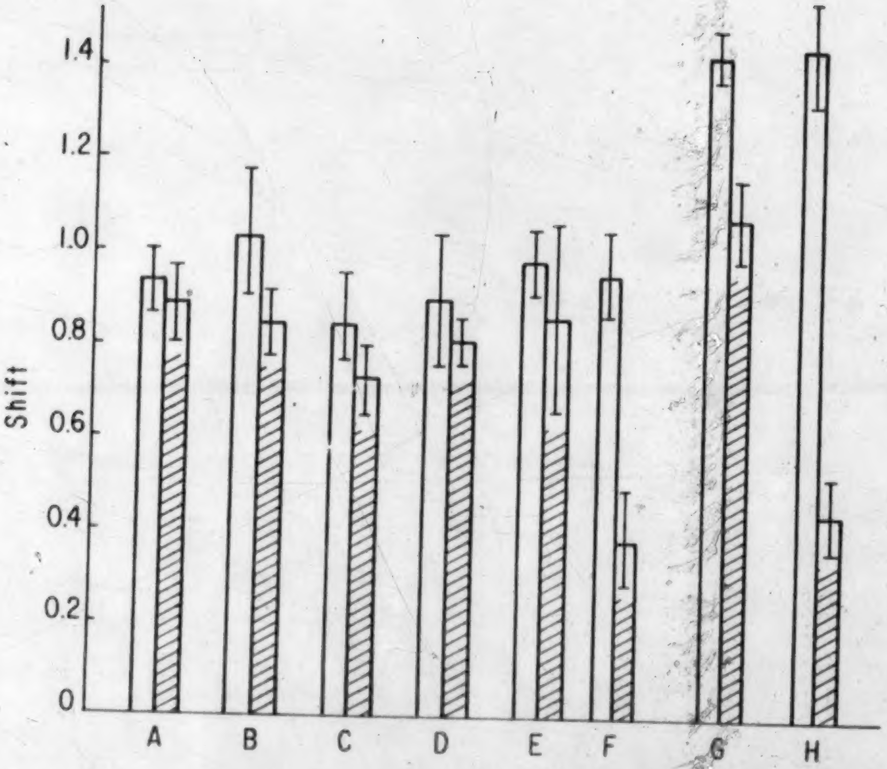


Figure 71. Histogram showing protecting with various agents against the phenoxybenzamine-induced shift of the dose-response curve. Magnitude of parallel shift (in log units) as ordinate. ☐ -Control tissues. ▨ - Protected tissues. Bars represent standard errors.

A - protected with 10^{-6}M histamine (N = 6)

B - protected with 10^{-4}M histamine (N = 6)

C - protected with 10^{-3}M histamine (N = 5)

D - protected with 10^{-5}M Et_2pyretamine (N = 4)

E - protected with 10^{-4}M Et_2pyretamine (N = 5)

F - protected with 10^{-3}M Et_2pyretamine (N = 4)

G - protected with 2 x 10^{-8}M diphenhydramine (N = 6)

H - protected with 10^{-7}M diphenhydramine (N = 4)

T. EFFECTS OF PROTECTING AGENTS ON THE PHENOXYBENZAMINE-INDUCED DEPRESSION OF MAXIMUM RESPONSE

The depression of the maximum response, by phenoxybenzamine, is much more sensitive to receptor protection by histamine. As seen in the histogram Fig. 72, even 10^{-6}M histamine produced a significant difference in the depression of maximum with higher doses providing greater degrees of protection. It is interesting to note that a subthreshold concentration of Et_2pyretamine (Fig. 70) caused a significant protection against depression of the maximum response by phenoxybenzamine but this result must be considered in light of the findings in Chapter III - Section V. This subthreshold concentration of Et_2pyretamine has no effect on the response to histamine (Fig. 73). Diphenhydramine provides extensive protection against depression of maximum response by phenoxybenzamine. The results of the statistical calculations are shown in Table VIII.

U. EFFECTS OF ANTAGONISM OF HISTAMINASE ON PROTECTION BY AGONISTS AGAINST PHENOXYBENZAMINE-INDUCED HISTAMINE RECEPTOR BLOCKADE

As mentioned in Chapter II - Section I (v), histamine is susceptible to degradation by the enzyme histaminase while Et_2pyretamine is not. The degradation of histamine, however, does not appear to be an important mechanism in terms of removal of agonist from the receptor locus, in this preparation. As can be seen in Fig. 74, a tissue exposed to a concentration of histamine sufficient to produce a maximum contraction, can sustain this response for 40 minutes. The small decrease in the contraction was due to desensitization as a higher dose of histamine did not increase the response.

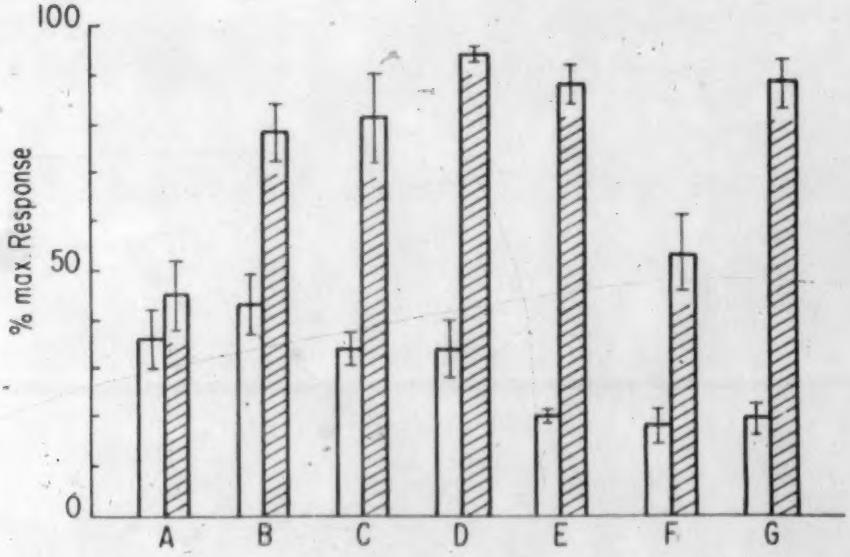


Figure 72. Histogram showing protection with various agents against the phenoxybenzamine-induced depression of the maximum response. Magnitude of the maximum response (after treatment with phenoxybenzamine 2 x 10^{-6}M for 3 min) in percent as ordinate. □ - Control tissues. ▨ - Protected tissues. Bars represent standard errors.

A - protection by 10^{-6}M histamine (N = 7)

B - protection by 10^{-4}M histamine (N = 7)

C - protection by 10^{-5}M Et_2pyretamine (N = 5)

D - protection by 5 x 10^{-5}M Et_2pyretamine (N = 3)

E - protection by 10^{-4}M Et_2pyretamine (N = 3)

F - protection by 2 x 10^{-8}M diphenhydramine (N = 4)

G - protection by 10^{-7}M diphenhydramine (N = 4)

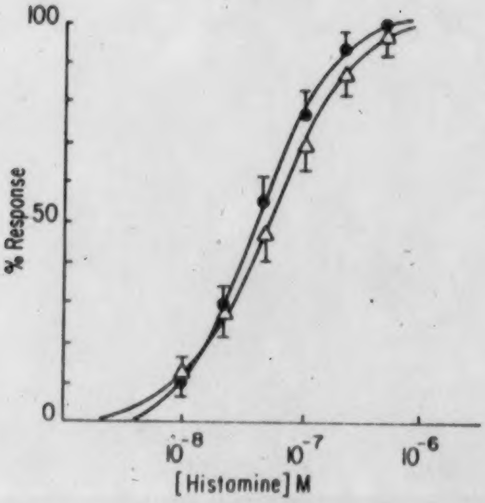


Figure 73. Effect of subthreshold dose of Et_2pyretamine on the response to histamine. Dose-response curve to histamine. Molar concentration as abscissa, percent maximum response as ordinate. N = 6. Bars represent standard errors.

●—● control dose-response curve

△—△ dose-response curve in the presence of Et_2pyretamine (10^{-5}M)

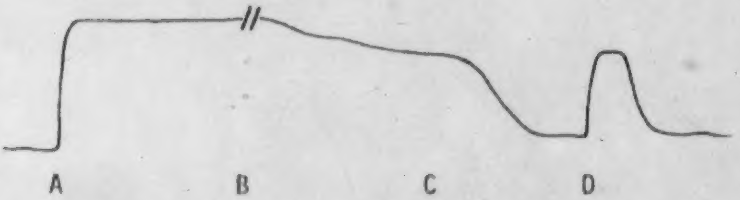


Figure 74. Sustained isotonic contraction by histamine

A - 10^{-6}M histamine

B - 35 minutes later

C - 10^{-5}M histamine

D - 5 x 10^{-5}M histamine

TABLE VIII

Protection by Agonists Against Phenoxybenzamine-Induced Depression of Maximum Response

Agent	n	Concentration	ΔMax*	Significant Difference from Control
Histamine	6	10^{-6}M	7.0 ± 2.0	$p < 0.01$
Histamine	7	10^{-4}M	35.3 ± 7.4	$p < 0.01$
Et_2pyretamine	5	10^{-5}M	46.8 ± 8.7	$p < 0.01$
Et_2pyretamine	3	5 x 10^{-5}M	60.0 ± 6.4	$p < 0.05$
Et_2pyretamine	3	10^{-4}M	68.0 ± 4.2	$p < 0.05$
Diphenhydramine	1	2 x 10^{-8}M	35.0 ± 6.2	$p < 0.01$
Diphenhydramine	1	10^{-7}M	69.0 ± 2.4	$p < 0.01$

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[*] Difference in the maximum responses between protected and non-protected tissues blocked with phenoxybenzamine.

Another protection experiment concerning the shift in the dose-response curve to histamine was also carried out in the presence of the histaminase inhibitor, hydroxylamine (10^{-4}M). As shown in Fig. 75, hydroxylamine had no effect on the response to histamine other than a slight potentiation as reported by Arunlakshana and coworkers (1954). There was no difference in the protecting effect of 10^{-6}M histamine in the presence or absence of hydroxylamine (Table VI).

V. CHEMICAL INTERACTION BETWEEN AGONISTS AND PHENOXYBENZAMINE

In light of the protection against depression of the maximum response by phenoxybenzamine observed with doses of Et_2pyretamine that had no visible receptor effect, the possibility of chemical scavenging of aziridinium ion by the partial agonist had to be considered. The assay described in Chapter II - Section I (iv) is sufficient to measure small differences in the aqueous concentration of Et_2pyretamine. Figure 76 shows the relation between aqueous concentration of the organic base and absorbance and it can be seen that it is linear, as predicted by the Beer-Lambert Law (Willard et al., 1965).

The percentages of phenoxybenzamine which react with various concentrations of Et_2pyretamine and thiosulphate ion, are shown in Fig. 77. A concentration of 5×10^{-5}M Et_2pyretamine left essentially no free aziridinium ion of phenoxybenzamine after 3 minutes under physiological conditions; the same is true for 10^{-2}M thiosulphate ion. These concentrations of "scavengers" for phenoxybenzamine would not necessarily prohibit blockade since there would be a competition, for phenoxybenzamine, between the receptor sites and the chemical reaction.

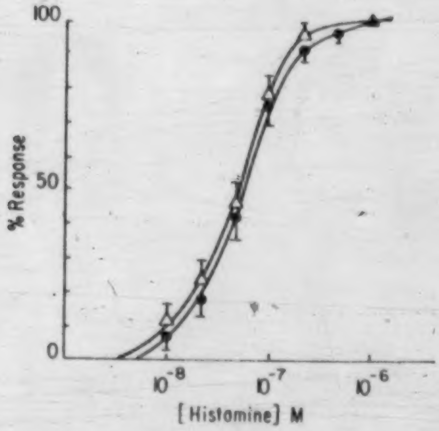

Figure 75. Effect of hydroxylamine (10^{-4}M) on the response to histamine. Dose-response curves to histamine. Percent maximum response as ordinate, molar concentration of histamine as abscissa. N = 4. Bars represent standard errors.

●—● control dose-response curve to histamine

△—△ response to histamine after hydroxylamine (10^{-4}M)

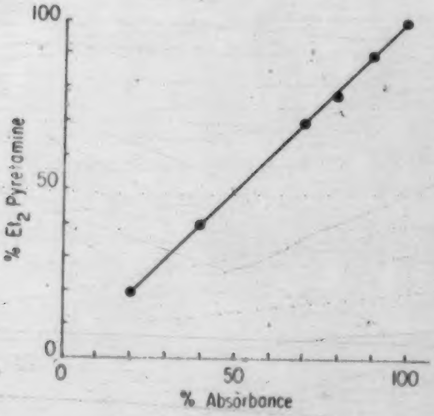

Figure 76. Relation between percent absorbance of solutions of Et_2pyretamine and percent Et_2pyretamine. Percent absorbance of dipicrylamine solution containing Et_2pyretamine as ordinate. Percent of 5 x 10^{-5}M Et_2pyretamine as ordinate.

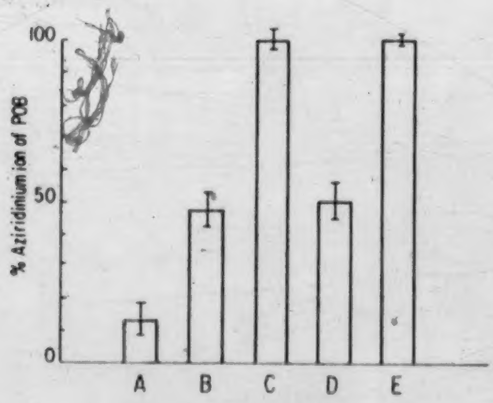

Figure 77. Percent phenoxybenzamine chemically interacting with either Et_2pyretamine or thiosulphate under pharmacological conditions (see text - Chapter III - Section V). Bars represent standard errors.

A - 10^{-5}M Et_2pyretamine (N = 4)

B - 2.5 x 10^{-5}M Et_2pyretamine (N = 4)

C - 5 x 10^{-5}M Et_2pyretamine (N = 4)

D - 5 x 10^{-3}M thiosulphate ion (N = 4)

E - 10^{-2}M thiosulphate ion (N = 4)

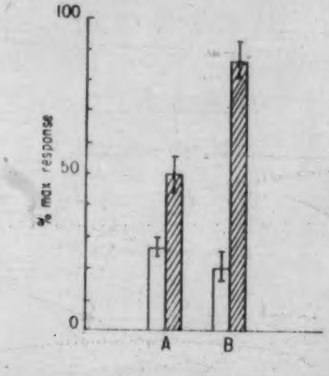

Figure 78. Differences in the depression of maximum response by phenoxybenzamine in the presence of thiosulphate ion. Bars represent standard errors. ☐ - Control tissues - blocked with phenoxybenzamine (2 x 10^{-6}M for 3 min) ▨-Tissues blocked with phenoxybenzamine in the presence of thiosulphate ion.

A - 5 x 10^{-3}M thiosulphate ion (N = 3)

B - 10^{-2}M thiosulphate ion (N = 3)

Figure 78 shows the effect of addition of the thiosulphate ion as a scavenger during phenoxybenzamine-induced blockade. Thiosulphate in a concentration sufficient to scavenge 100% of the phenoxybenzamine in 3 minutes (10^{-2}M), produced a difference in the phenoxybenzamine-induced depression of the maximum response (from that of a control tissue exposed to phenoxybenzamine in the absence of thiosulphate) of approximately 66%. It thus becomes evident that the 46.8% value for the difference in the phenoxybenzamine-induced depression of the maximum response observed in the presence of 10^{-5}M Et_2pyretamine (subthreshold - Fig. 70, Fig. 73) is most likely an artifact due to chemical interaction of the agonist with phenoxybenzamine. All protection effects by this agonist must therefore be considered to be partly due to chemical effects and partly to receptor protection. A difference of 0.33 log units is observed when phenoxybenzamine is used to induce a parallel shift in the dose-response curve in the presence of 10^{-2}M thiosulphate ion. This again must be considered in the light of the results in Fig. 71 where Et_2pyretamine, in a very high concentration, (10^{-3}M) produces a shift of 0.57 ± 0.1 log units.

Histamine (10^{-4}M) does not appear to react to a significant extent with phenoxybenzamine. The selective protection by low concentrations against depression of maximum response by phenoxybenzamine is therefore most likely due to receptor protection effects and not chemical scavenging. It is interesting to note that even 10^{-3}M histamine caused no difference in the shift in dose-response curve with phenoxybenzamine. This would be unlikely if the agonist could interact with phenoxybenzamine to any significant degree. These results are summarized in Table IX.

TABLE IX

Chemical Interaction Between Phenoxybenzamine and Scavenging Agents

Agent	Concentration	n	% Phenoxybenzamine Interacting with Agent	Remaining Phenoxybenzamine (%) (approx)
Et_2pyretamine	10^{-5}M	4	13 ± 7	87
Et_2pyretamine	2.5 x 10^{-5}M	4	47 ± 7	53
Et_2pyretamine	5 x 10^{-5}M	4	100 ± 4	0
Thiosulphate	5 x 10^{-3}M	4	50 ± 6	50
Thiosulphate	10^{-2}M	4	100 ± 2	0
Histamine	10^{-4}M	4	0	100

IV DISCUSSION

A. REVIEW OF RESULTS

At this point it is useful to summarize the major results outlined in this thesis before beginning a discussion on their significance.

1. A maximum parallel shift of the dose-response curve of 0.67 ± 0.07 log units is observed when the preparation is blocked with phenoxybenzamine but washed with Tyrode solution not containing thiosulphate ion. Further treatment with phenoxybenzamine results in a dose-response curve shifted less than 2 log units to the right but with a depressed maximum response (Chapter III - Section C). This result was obtained isotonically, but is similar to results in the literature which were obtained isometrically.
2. The β-haloalkylamine ECB provides an estimate of the receptor reserve that is significantly different from that provided by either SY14 or phenoxybenzamine. The agents ECB SY14 and phenoxybenzamine all cause maximum shifts of the dose-response curves which are significantly different (Chapter III - Section F).
3. Very high doses of histamine fail to protect against the phenoxybenzamine-induced shift of the dose-response curve. The depression, by phenoxybenzamine, of the maximum response is very sensitive to protection with histamine (Chapter III - Sections S and T).

4. Washing a tissue blocked with phenoxybenzamine with Tyrode solution containing thiosulphate ion or BSA and thiosulphate ion, selectively reverses the receptor-specific depression of the maximum response but not the phenoxybenzamine-induced shift of the dose-response curve. The same can be achieved when the tissue is pretreated with mercaptopyruvate (Chapter III - Sections G and H).

5. The aziridinium ion of the β-haloalkylamine DMPEA appears to be a muscarinic agonist in this preparation. This agent also selectively antagonizes the phenoxybenzamine-induced depression of the maximum response but not the shift of the dose-response curve. This is achieved by pretreatment of the tissue with concentrations of DMPEA that do not affect the histamine response (Chapter III - Sections J and L).

6. Desensitization appears to selectively "protect" against the phenoxybenzamine-induced depression of the maximum response. Although maximal desensitization has a small effect on the phenoxybenzamine-induced shift of the dose-response curve, this effect cannot be correlated with the level of desensitization (Chapter III - Sections N and O).

7. The partial agonist Et_2pyretamine, used to protect against blockade by phenoxybenzamine, can significantly react with the aziridinium ion of phenoxybenzamine. Such chemical interactions must be differentiated from pharmacological effects (Chapter III - Section V).

There are a number of hypotheses available to explain these findings. The phenomenon of the parallel shift of the dose-response curve by these agents can be explained by competitive reversible blockade, the presence of spare cells, the presence of spare receptors or the existence of more than one binding site for the β-haloalkylamine. It will be seen that the latter two hypotheses are the major ones that concern this thesis but all must be discussed.

B. COMPETITIVE REVERSIBLE BLOCKADE

A parallel shift of a dose-response curve with retention of the maximum response is normally indicative of competitive reversible blockade. Whereas competitive reversible blockade is normally diminished by washing, it was found in these studies (as well as in the original experiments by Nickerson in 1956) that prolonged washing did not significantly reverse the blockade (Fig. 50). The obvious candidate for reversible blockade would be the aziridinium ion of the β-haloalkylamine. An example of a situation where an aziridinium ion was found to be responsible for a seemingly irreversible parallel shift of the dose-response curve was in the blockade of noradrenaline by SY28 in rat vas deferens (Moran, Triggle and Triggle, 1969). It was found by these workers that in the presence of thiosulphate ion the parallel shift of the dose-response curve disappeared. Thus the finding in rat vas deferens did not require any explanation more complicated than simple competitive blockade by the aziridinium ion of SY28. In our preparation, however, the phenoxybenzamine-induced shift of the dose-response curve was not reversed by high concentrations of thiosulphate ion. The shift of the dose-response curve in the longitudinal smooth muscle of the guinea pig ileum must then be considered to be the result of a truly irreversible antagonism presumably involving a covalent receptor-antagonist complex.

C. THE SPARE CELL HYPOTHESIS

It has been proposed that "spare cells" are responsible for the β-haloalkylamine-induced shift of the dose-response curve with retention of maximum response (Waud, 1968). It is difficult to imagine how there could be spare cells when measurement of response are carried out isometrically since the total force produced by the muscle would appear to depend on the sum of the forces generated by each cell. Under these circumstances, it would be expected that irreversible inactivation of any portion of the smooth muscle cells would cause an immediate depression in the isometrically recorded maximum response. It was found by Cook (1971), however, that phenoxybenzamine produced a parallel shift in the dose-response curve with retention of maximum response (measured isometrically). For the "spare cell" hypothesis to be valid, there must be some other mechanism limiting the mechanical response (in the case of isometric studies).

The "critical cells" (some fraction of the total population) would be defined as the minimum number of cells required for maximum response. Under these circumstances it might be expected that the magnitude of this shift of the dose-response curve would not be the same when measured isotonically and isometrically. If the smooth muscle cells are oriented such that they contract in series, then it might be possible for maximum isotonic shortening to occur (providing that we are dealing with the "critical fraction" of muscle cells).

If, however, these cells are arranged in parallel, then although maximum isometric force may still be achieved, isotonic shortening would be greatly diminished. Thus it can be seen that the orientation of the muscle cells would affect whether or not the whole tissue would achieve maximum isotonic shortening. The maximum phenoxybenzamine-induced shift of the dose-response curve as measured isotonically (Fig. 38) was very similar to that measured isometrically (Cook, 1971) when thiosulphate ion was not present in the wash medium. These data indicate that very similar "receptor reserves" are measured by these different methods. In terms of the "spare cell" hypothesis, it must therefore be assumed that the same number of cells required for maximal shortening is about equal to the same number of cells required for the generation of maximal force of contraction. This is untenable in the light of the earlier discussion, thus the experiments shown in Fig. 38 and those of Cook do not tend to support the "spare cell" hypothesis.

A basic tenet of this hypothesis is that the "spare cells" are located deeper in the tissue and are therefore not as susceptible to blockade by β-haloalkylamine. Under these circumstances, it might be supposed that long exposure times to a dose of antagonist sufficient to produce a shift in the dose-response curve under normal conditions (3 minute exposure) should produce depression of the maximum response. The increase in time period of exposure would enable the antagonist to penetrate to the "spare cells" and cause depression of the maximum response. Many variations of concentrations of antagonist and times of exposure were utilized in the studies comparing the maximum β-haloalkylamine-induced shift of the dose-response curve obtained for different agents. In these studies, various doses of β-haloalkylamine were exposed to the tissues for rather long exposure times. It was found that longer exposure times were not the deciding factor in whether or not the maximum response was depressed but that concentration appeared to be the sole determinant. In experiments utilizing a longer time of exposure of the antagonist to the tissue, there was sufficient blocking agent in the medium at the end of the incubation time, to cause blockade of a fresh tissue. This implies that there was a substantial concentration of aziridinium ion present in the medium and that the antagonist was not simply being "sequestered" by the outside muscle cells. In light of these results, "spare cells" do not appear to be operative in this preparation.

D. THE SPARE RECEPTOR HYPOTHESIS

It would be expected that irreversible inactivation of any part of a population of receptors would immediately depress the maximum response if activation of the total population of receptors was required for the achievement of the maximum response to the agonist. The irreversible parallel shift with retention of maximum response could therefore not be accounted for by the existing theories of drug-receptor interactions before Stephenson (1956) and Nickerson (1956) independently proposed the "receptor reserve" hypothesis. The actual theory describing this "receptor reserve" has been discussed in Chapter I - Section G and need not be elaborated upon further. An analysis of the predicted behaviour of a preparation supposedly possessing such a "receptor reserve" is a useful starting point from which to consider the merits of this theory.

Nickerson (1956) estimated the receptor reserve for histamine in guinea pig ileum to be 99%. The magnitude of this quantity, as defined, is a unique property of the preparation and therefore independent of whatever agent is used to measure it, yet it can be seen in Chapter III - Section F that two agents measure significantly different receptor reserves (ECB, SY14, and phenoxybenzamine). It should be noted that the blockade of the last 10% of the receptors results in the extension of the parallel shift in the dose-response curve of one complete log unit (Chapter II - Section F). Measurement of the extent of blockade of the receptors in this range (90% to 100%) is relatively accurate since small differences in the number of receptors irreversibly inactivated are reflected by large differences in the parallel shift of the dose-response curve. It therefore is difficult to attribute the differences in the receptor reserve estimates to errors of measurement.

The lack of protection afforded by a dose of histamine, capable of eliciting a maximum response in an unblocked tissue, against the irreversible shift in the dose-response curve (Fig. 71), can be explained by the spare receptor hypothesis. It must be remembered that irreversible antagonists, by definition, possess a relatively small rate of dissociation from the receptor while this need not necessarily be true of full agonists (Paton, 1961). Thus, β-haloalkylamines possess a "thermodynamic advantage" over agonists when both compete for a common binding site or binding sites that are not mutually exclusive (linked allosterically). An excess of agonist would thus be required to overcome this effect and when a concentration of histamine known to occupy 100% of the receptor sites is used to protect against blockade by irreversible antagonist, complete protection should not be expected. If it is assumed that 99% of the histamine receptors are "spare" and only 1% need be activated to achieve maximum response, the dose of histamine which would elicit this maximum tissue response would only be occupying 1% of the receptors. It should therefore not be assumed that this dose of histamine would cause any protection against the phenoxybenzamine-induced irreversible shift of the dose-response curve as this shift would reflect blockade of the 99% of the receptors not occupied by agonist (Ariëns et al.,1960; Waud, 1962). As the protecting dose of full agonist is increased, however, there would a greater protection of the "spare receptors" thus a concentration of histamine that was 100 times that which is required for maximum response should cause some measure of protection against the shift of the dose-response curve. As seen in Table VII and Fig. 71, however, concentrations of up to 100 times that which is required for maximum response do not cause a signifigant difference in the amount of phenoxybenzamine-induced shift of the dose-response curve. This implies that either the "receptor reserve" is greater than 99.9% (and that the double-reciprocal plot used to calculate this quantity is not valid) or that some other mechanism of blockade is operative.

If the 2 log unit shift of the dose-response curve and subsequent depression of the maximum response caused by β-haloalkylamines is the result of the sequential alkylation of the same population of histamine receptors, then under no circumstances should there occur a depression of the maximum response of a dose-response curve that has not been shifted 2 log units to the right. As can be seen in Fig. 38, however, when thiosulphate ion is not present in the bathing medium of a tissue blocked with phenoxybenzamine, the dose-response curve of this tissue is not shifted a full 2 log units to the right yet the maximum response is depressed. This presents a rather incongruous situation in which only approximately 96% of the receptors are alkylated by phenoxybenzamine before depression of maximum response occurs under these wash conditions and 99% of the receptors become alkylated before depression of maximum response when thiosulphate ion is present in the wash medium. A better illustration of this effect was given by Cook (1971) in studies showing only a 1 log unit shift, by phenoxybenzamine, of the dose-response curves to histamine before depression of the maximum response. Ariëns and coworkers (1960) also have shown a dose-response curve with a depressed maximum response that is shifted only 1.33 log units to the right after treatment with dibenamine.

In terms of the "spare receptor" hypothesis, the depression of the maximum response to hitamine in guinea pig ileum is, by definition, the result of blockade of the remaining 1% of the receptors. This depression of maximum response to histamine appears to be receptor-specific as the responses to potassium (Fig. 36) and barium (Fig. 37) are not affected by treatment of the tissue with concentrations of β-haloalkylamine sufficient to all but abolish any response to histamine (Fig. 34).

The selective reversal of the phenoxybenzamine-induced depression of the maximum response, by various treatments of the tissues, is difficult to explain in terms of a "receptor reserve". As seen in Fig. 47, the incubation of a tissue, previously treated with phenoxybenzamine (maximum response is depressed to 72%), in Tyrode solution containing thiosulphate ion causes reversal of a large portion of the depression of the maximum response (blockade reverses, maximum response becomes 97% of control). As thiosulphate can only react with aziridinium ion, and not covalently-bound β-haloalkylamine, this implies that the observed phenoxybenzamine-induced depression of the maximum response is the result of an aziridinium ion-receptor complex.

There is evidence to suggest that the aziridinium ion of phenoxybenzamine is bound very strongly to the receptor. The $t_{1/2}$ for the recovery of the maximum response of a tissue blocked with phenoxybenzamine is long ($t_{1/2}$ =1150). Although thiosulphate ion increases the rate of this recovery process, the reaction between receptor-bound aziridinium ion and thiosulphate ion in aqueous buffer (see chapter III-Section I) . Furthermore, the rate of hydrolysis of the receptor-bound aziridinium ion appears to be much slower than for free aziridinium ion. As seen in Fig. 54, after 3 hours in aqueous buffer, there is only 25% detectable aziridinium ion, the remainder having hydrolyzed. The blockade of the maximum response, however, reverses very little in an equivalent time period indicating minimal loss of this antagonist species to the process of hydrolysis. These data indicate that the bound aziridinium ion has a much lower chemical reactivity than the free species. The extent of this "resistance" to chemical reaction cannot be properly estimated since the actual amount of bound materail is unknown.

Although thiosulphate ion increased the rate of dissociation of aziridinium ion from the receptor, only rarely was this treatment sufficient to reverse a severely depressed maximum response (<20%) to values approaching 100% of control. With BSA in the wash medium as well as thiosulphate ion, this was readily achieved, the $t_{\frac{1}{2}}$ for reversal of the depression of maximum response being increased almost 6-fold ($t_{\frac{1}{2}}$ =200 min). BSA has been used to remove the disulfonic acid stilbene derivative (SITS) previously thought to be covalently bound to the red blood cell membrane (Cabantchik and Rothstein, 1972). The fact that BSA binds the aziridinium ion of phenoxybenzamine is not surprising since this species could theoretically alkylate a portion of the amino acid side chains in the protein. Drug-protein binding interactions not involving alkylation are also well known (Goldsteing, 1949). The need for the presence of BSA in the wash medium for the achievement of complete recovery of the maximum response further illustrates the tight-binding and low chemical reactivity of the receptor-bound aziridinium ion.

If the depression of the maximum response was the result of the same mechanism producing the irreversible shift of the dose-response curve then it would be expected that a process which reverses the depression of the maximum response would also reverse a portion of the shift. The reversal of blockade from any part of the "receptor reserve" would be reflected in a reversal of a portion of the irreversible shift of the dose-response curve. Any increase in the number of unblocked receptors in a tissue, under circumstances where the dose-response curve is shifted 2 log units, would cause a measureable reversal of this shift as small changes in the number of blocked receptors are reflected by large changes in the shift. As a reversal of the shift of the dose-response curve was never achieved with these wash procedures, there appears to be a dichotomy in the characteristics of the receptors mediating this shift and those mediating depression of the maximum response.

It is possible that the very slow reversal of the depression of the maximum response ($t_{\frac{1}{2}}$ = 1150 min) reflects not a removal of tightly bound aziridinium ion from the receptor but the slow hydrolysis of a covalent bond (between phenoxybenzamine and the receptor) and a replenishing of blockade from a nearby pool of blocking agent. The thiosulphate and BSA could then be "scavenging" antagonist thus preventing further alkylation of free receptors and giving the appearance of an acceleration in the reversal of blockade. It has been observed, however, that any existing pool of antagonist has no detectable effect after 2 hours. All data concerning the reversal of blockade had been determined after a 150 minute wash period making it unlikely that such a mechanism would have any effect on blockade. Since BSA and thiosulphate ion accelerate reversal of blockade 5 and 6 hours after the initial exposure to phenoxybenzamine, it is much more likely that non-alkylated aziridinium ion was being removed from the receptor or a receptor-linked binding site.

Treatment of a tissue with mercaptopyruvate also selectively reverses the blockade of the maximum response by phenoxybenzamine (Fig. 52). The effect is qualitatively similar to that produced by wash with Tyrode solution containing BSA and thiosulphate ion in that no reversal of the shift of the dose-response could be achieved. A further discussion of the effects with mercaptopyruvate follows (vide infra).

As the "spare receptor" hypothesis states that the depression by β-haloalkylamines of the maximum response is caused by an extension of the same mechanism that causes the shift in the dose-response curve (the sequential alkylation of receptors) it does not provide an explanation for the present results.

I. THE SHIFT OF THE DOSE-RESPONSE CURVE AND DEPRESSION OF MAXIMUM RESPONSE CONSIDERED AS SEPARATE PROCESSES

There appears to be a divergence in the properties of the receptors mediating the β-haloalkylamine-induced shift of the dose-response curve and the depression of the maximum response. Some of the results suggesting this divergence have been discussed previously and need not be reviewed in detail.

While histamine protects against the phenoxybenzamine-induced depression of the maximum response with facility, even in high doses, it affords little protection against the phenoxybenzamine-induced shift of the dose-response curve. Although the "spare receptor" hypothesis predicts this behavior to a certain degree (histamine protects the 1% of the receptors required for the maximum response more easily than the other 99%), it does not account for such a large dichotomy in the protection afforded by histamine against the phenoxybenzamine-induced shift of the dose-response curve and depression of maximum response as has been observed (Figs. 71, 72). Separate mechanisms for the two stages of blockade observed with phenoxybenzamine (shift of the dose-response curve and depression of the maximum response) are also implied by the fact that various treatments selectively reverse the phenoxybenzamine-induced depression of the maximum response but not the shift of the dose-response curve.

DMPEA further differentiates these two stages of blockade. Except at high concentrations, this agent causes no significant difference in the response to histamine. It must therefore be assumed that DMPEA, although theoretically capable of alkylating electrophilic sites on the receptor, does not interfere with the process of activation of the H_1 receptor by histamine. The reason probably lies in the unusual chemical structure of this agent. If the chemical structure has any role to play in the initial binding of the aziridinium ion, as there is sufficient reason to believe, then the absence of the methylene bridge between the aromatic ring and the nitrogen atom might well give DMPEA a different specificity with respect to the histamine receptor.

As seen in Chapter III - Section L,pretreatment of the tissue with DMPEA, at this concentration, does little to affect the phenoxybenzamine-induced shift of the dose-response curve. This agent does, however, significantly protect against the phenoxybenzamine-induced depression of the maximum response. The protection in this case does not refer to DMPEA acting as a competitive reversible agent competing with the phenoxybenzamine for a common binding site but rather to an alkylation of some site by DMPEA which in some way impedes the binding of the aziridinium ion of phenoxybenzamine to the "depression" site. This protection cannot be attributed to the chemical reaction of DMPEA with phenoxybenzamine to form piperazinium compounds, as the tissue was washed for 20 minutes between the initial exposure to DMPEA and the treatment with phenoxybenzamine. It is unlikely that the aziridinium ion of DMPEA alkylates an electrophilic group which would otherwise be alkylated by phenoxybenzamine as the DMPEA pretreatment does not preclude but only antagonizes depression of the maximum response by phenoxybenzamine. As stated earlier, the depression by phenoxybenzamine of the maximum response is probably not due to an alkylation of the receptor making the possibility of a competition between this compound and DMPEA for the same electrophilic chemical group even more unlikely.

Desensitization appears to have a much greater effect on the phenoxybenzamine-induced depression of the maximum response to histamine than on the shift of the dose-response curve. There is a reasonably good correlation between the amount of desensitization induced in a preparation prior to blockade and the antagonism produced by phenoxybenzamine (Fig. 65) against the maximum response. No such correlation is evident for the blockade by phenoxybenzamine resulting in the shift of the dose-response curve, thus desensitization appears to further differentiate these two stages of blockade. There is some overlap in this case, however, since maximal desensitization produces a small but statistically significant difference in the phenoxybenzamine-induced shift of the dose-response curve.

Thus the results of the receptor protection studies, the selective reversal of the phenoxybenzamine-induced depression of the maximum response, the selective protection by DMPEA and the differences in the effects of desensitization all suggest that these two stages of blockade are mediated by separate mechanisms. These two mechanisms may reflect binding by β-haloalkylamine to two distinct populations of receptors, a large proportion of which are spare, binding of the antagonist to two separate receptor-associated sites or the binding of β-haloalkylamine to perturb an existing equilibrium between two oligomeric forms of the receptor. These latter two possibilities have much in common and need not be mutually exclusive but the hypothesis describing two distinct receptor forms and a receptor-reserve is a modification of the "spare receptor" theory and requires separate discussion.

F. TWO OR MORE POPULATIONS OF RECEPTORS WITH DIFFERING SENSITIVITY TO β-HALOALKYLAMINES

It is possible to account for some of the dichotomy concerning the phenoxybenzamine-induced shift of the dose-response curve and the depression of the maximum response in terms of two populations of receptors with differing sensitivities to blocking agent. Spare receptors would have to be invoked, however, to explain the 2 log unit shift of the dose-response curve with retention of maximum response. In terms of this hypothesis, one population of receptors would not be alkylated by β-haloalkylamine but only bind the aziridinium ion. Thus, the selective reversal of the phenoxybenzamine-induced depression of the maximum response would reflect removal of aziridinium ion from this population of receptors. DMPEA would also affect this population (causing the selective protection against phenoxybenzamine-induced depression of the maximum response) and not the other.

Finally, the differences in antagonism by phenoxybenzamine in the presence of desensitization could also be explained by postulating that the two populations of receptors are not affected by desensitization in an identical manner and that this difference produces different effects on antagonism by phenoxybenzamine. In this way, the hypothesis is able to avoid some of the inconsistencies encountered when discussing these experimental findings in terms of the "spare receptor" hypothesis.

It should be noted, however, that the hypothesis describing two separate populations of receptors requires assumptions as to the reactivity of the two populations of receptors to β-haloalkylamine, the selective binding of DMPEA and the behavior of these two populations of receptors with respect to desensitization.

There are some experimental results, however, which are difficult to explain in terms of this hypothesis. It is possible to account for the differences in protection afforded by histamine only by postulating that while β-haloalkylamine can antagonize one population of receptors (by presumably alkylating a site distinct from that occupied by histamine), the protecting dose of histamine prevents antagonism of the other population of receptors. It should be noted, however, that additional hypotheses would then have to be invoked to explain the mechanism by which phenoxybenzamine is able to alkylate the one population of receptors in the presence of such high concentrations of histamine.

If, in this scheme, activation by histamine of any 1% of the receptor population resulted in maximum tissue response then any β-haloalkylamine, under some experimental circumstances, should produce the same shift of the dose-response curve before depression of the maximum response. This would arise because irrespective of whether the site was alkylated or merely subject to tight binding of aziridinium ion, under some experimental conditions the appropriate critical fraction of receptors would remain free thus resulting in the maximum parallel shift (2 log units). The maximum shift is still a property of the system, and thus the finding of different maximum shifts of the dose-response curve is not amenable to ready explanation.

It is possible to explain different magnitudes of parallel shift of the dose-response curve by assigning different roles, in terms of the excitation-contraction mechanism, to the receptors. Thus, activation of any 1% of the receptors would yield maximum response but blockade of any portion of the "non-alkylatable" population would cause depression of the maximum response. Blockade of various proportions of the 99% of the "alkylatable receptors" would cause shifts of the dose-response curve (as in the "spare-receptor" hypothesis - Chapter I - Section G). Under these circumstances, blocking agents with a higher affinity for the "non-alkylatable" receptors could depress the maximum response before saturating the "alkylatable" receptors thereby producing dose-response curves not shifted 2 log units but with a depressed maximum response. These added assumptions, concerning the lack of protection with histamine of the "alkylatable" receptors and the differing roles with respect to contraction make this hypothesis if not improbable, at least complex and a number of assumptions are involved for which there is no pharmacological precedence. A similar hypothesis, however, proposing two distinct β-haloalkylamine binding sites associated with one receptor presents an attractive alternative. Inherent in this hypothesis are explanations for the dichotomy in the receptor-protection observed with histamine and the maximum β-haloalkylamine-induced shifts of the dose-response curve of differing magnitude.

G. EXPLANATION OF RESULTS IN TERMS OF TWO BINDING SITES FOR β-HALOALKYLAMINES

The preceding discussion outlines how many of the experiments, dealt with in this thesis, indicate that separate mechanisms may be responsible for β-haloalkylamine-induced shift of the dose-response curve and the subsequent depression of the maximum response. Another way of explaining this dichotomy is to propose two separate binding sites for the mediation of these two stages of blockade. Precedents already exist for the concept of two β-haloalkylamine binding sites on one receptor in the α-adrenergic receptor system (Moran, Swamy, and Triggle, 1970; Janis and Triggle, 1971; Swamy and Triggle, 1972) and the acetylcholine receptor system in rat jejunum (Moran and Triggle, 1970).

This hypothesis, as applied to the histamine H_1 receptor is a modification and extension of the two-site hypothesis put forward by Moran and Triggle (1970) for the acetylcholine receptor. Moran and Triggle suggested that in terms of this hypothesis the acetylcholine receptor consists of a binding site for the quaternary head of all agonists and two adjacent sites to which bind full agonists (such as acetylcholine) and partial agonists respectively. This latter site is alkylated by low doses of β-haloalkylamine, the result being the depression of the maximum response to partial agonists (the binding of partial agonists would be prohibited by the presence of the β-haloalkylamine) and the allosteric perturbation of the acetylcholine binding site. This allosteric perturbation would result in a decreased affinity of the receptor for acetylcholine. This would cause a shift of the dose-response curve with retention of the maximum response. At higher doses, the β-haloalkylamine would block the common binding site which would then produce a decrease in the maximum response to all agonists. The two β-haloalkylamine binding sites could therefore be described as the "shift" site (binding of antagonists at this site results in a shift of the dose-response curve to full agonists) and a "depression" site (binding of the antagonist at this site results in the depression of the maximum response to all agonists).

There is no reason to suppose that the two binding sites for β-haloalkylamines associated with the histamine H_1 receptor bear any relation to the sites described in the Moran and Triggle (1970) hypothesis. It is logical, however, to assign the mediation of the shift of the dose-response curve to one site and the depression of the maximum response to the other in light of the results implying the different nature of these two phenomena. For convenience, therefore, the terms "shift" site and "depression" site will be used to describe the two β-haloalkylamine binding sites for the histamine receptor.

i) The Shift of the Dose-Response Curve

The alkylation of the "shift" site would result in the production of up to a 2 log unit shift in the dose-response curve. This site would appear to be separate from the binding site for histamine since very large doses of histamine fail to affect alkylation of this site by antagonists. As there is no need to invoke spare receptors in this scheme, there would be no reason to suppose that the concentration of histamine which produced the maximum response was not occupying most if not all of the receptors. The fact that 100 times this dose of histamine does not affect the β-haolalkylamine-induced shift in the dose-response curve indicates that a separate binding site, from that for histamine, most likely mediates this process. Since partial agonists are perhaps somewhat better agents for protecting against the β-haloalkylamine-induced shift in the dose-response curve, there could exist a similarity between the "shift" site in the Moran and Triggle two-site hypothesis and the "shift" site for the histamine receptor. This data, however, cannot be considered unequivocal in light of the agonist-antagonist chemical interactions described in Chapter III - Section V.

In terms of the Moran and Triggle hypothesis, the allosteric perturbation of the receptor results in a decrease of the affinity of the drug for this receptor. If the alkylating agent causes the "shift" of the dose-response by decreasing the affinity of the drugs for the receptor with no concomitant effects on the ability of the stimulated receptor to cause contraction, then in the absence of spare receptors it would be expected that dose-response curves shifted 1 log unit or less would differ in slope from the control curve. The curve for the blocked preparation might be expected to be skewed such that, at low doses of agonist, the remaining receptors with normal affinity for histamine would be activated and the dose-response curve in this region would resemble that of the control.

As the maximum response is approached, however, the agonist would have to stimulate the receptors with a decreased affinity for histamine thus, higher concentrations would have to be employed to achieve maximum response. In this way, a smaller slope than control would be expected in this region of the dose-response curve. As seen in Fig. 39, however, the slopes of the shifted curves appear to be the same as the control curve (with the exception of the maximally shifted curve which has a slope that is greater than control) thus the alkylation process appears to produce a dose-response curve which is parallel to the control. This would indicate that the allosteric perturbation caused by β-haloalkylamines produces the shift of the dose-response curve by impeding the mechanism by which a stimulated receptor initiates contraction and not by interfering with the actual binding of histamine to the receptor.

As stated earlier, there is sufficient evidence to imply that the "shift" site is alkylated by β-haloalkylamines. This site appears to be alkylated with great facility since the shift of the dose-response curve can be achieved with exposures of tissue to β-haolalkylamine for very short time periods. An irreversible shift of the dose-response curve can also be produced by incubation of tissue with β-haloalkylamine in the presence of high concentrations of thiosulphate ion.

ii) The "Depression" Site

There is very little data, at present, capable of providing a clue as to the nature of the "depression" site. Binding of antagonist to this site, by definition, causes depression of the maximum response. It is tempting to assume that the histamine binding site and the "depression" site are one and the same but at present the data which suggests this is inconclusive. The protection afforded by various doses of histamine parallels the pharmacological effect of these doses of agonist. The lowest dose of agonist which can elicit the maximum response (and therefore presumably stimulate most if not all of the receptors assuming no receptor reserve) is capable of causing a significant difference in the phenoxybenzamine-induced depression of maximum response (Fig. 72). The fact that this antagonist-induced depression of maximum response is so sensitive to protection with relatively low doses of histamine (with regard to protection studies in general) indicates that either the "depression" site is the binding site for histamine or that the binding of histamine to the receptor induces an allosteric effect on the "depression" site to antagonize the binding of β-haloalkylamine.

It is possible that the histamine binding site and "depression" site are allosterically linked. Although this would complicate the hypothesis (perhaps needlessly at this point) it cannot be ignored.

Although the bond between the "depression" site and the β-haloalkylamine is strong ($t_{1/2}$ = 1150 min for reversal of blockade), it does not appear to be covalent in nature. The selective reversal of the phenoxybenzamine-induced depression of the maximum response indicates the reversible nature of the antagonism due to binding of blocking agent at the "depression site". It is interesting to note that while the "depression" site described for the histamine receptor and the binding site for quaternary ammonium moieties of agonists are qualitatively similar in that binding of β-haloalkylamine to the latter group also produces depression of the maximum response to full agonists, there are also differences. If coulombic attraction is to play a role in the binding of agonist to receptor, as there is reason to believe, then the binding site for the quaternary head of agonists would possess a negatively charged chemical group which in the case of the acetylcholine receptor is alkylated by the electrophilic aziridinium ion of the β-haloalkylamine. The "depression" site for the histamine receptor may possess a similar chemical group (which attracts the positively charged aziridinium ion) but either it may not be of suitable reactivity or the β-haloalkylamine binding site may not allow the proper binding of aziridinium ion to allow a covalent bond to be formed.

DMPEA appears to interfere with the binding of phenoxybenzamine to the "depression" site. As stated earlier, pretreatment of the preparation with DMPEA does not preclude phenoxybenzamine-induced depression of the maximum response and a possible mechanism for this effect would be the partial blockade, by DMPEA, of a portion of the receptor required for the binding of phenoxybenzamine. It is possible that the DMPEA, a much more reactive alkylating agent than phenoxybenzamine, alkylates to a suitable chemical group and partially occludes the "depression" site. It is interesting to note the lack of effect of DMPEA pretreatment on the response to histamine. Although this could be interpreted as evidence that the "depression" site and the binding site for histamine are separate, there is no *a priori* reason to suppose that all of the phenoxybenzamine molecule binds solely to the histamine binding site. As there could be some overlap to other portions of the receptor macromolecule, antagonism of binding to the "depression" site need not be accompanied by antagonism of histamine binding.

iii) Concurrent Binding of β-Haloalkylamines to Both Sites

One of the greatest advantages of this hypothesis is the ability to account for parallel shifts in the dose-response curves of different magnitude for different antagonists. The magnitude of the parallel shift would thus not reflect the blockade of the receptor-reserve, in terms of this hypothesis, but rather the differential affinities of the β-haloalkylamine to the two separate sites. A β-haloalkylamine such as ECB might possess a greater affinity for the "depression" site than does an agent such as phenoxybenzamine. ECB would then bind to the "depression" site before all of the "shift" sites are saturated, thereby producing a depressed maximum response that is accompanied by a smaller shift than for phenoxybenzamine. When dealing with two separate chemical reactions with differing rates (the binding of β-haloalkylamines to the two different sites with differing K_a's) it would not be expected that one reaction could be brought to completion (binding of antagonist to "shift" site to cause 2 log unit shift) before initiation of the other (binding of any blocking agent to "depression" site). Thus the 1.53 log unit shift of the dose-response curve represents the maximum differentiation of these two binding processes for ECB in the sense that the maximum number of "shift" sites are not alkylated before depression of the maximum response occurs. For phenoxybenzamine, an agent with apparently a greater affinity for the "shift" site or a smaller affinity for the "depression" site than ECB, the maximum differentiation between these two binding processes is greater and a 2 log unit shift of the dose-response curve can be achieved before binding of antagonist to the "depression" site. The magnitude of the parallel shift would thus depend on the chemical structure of each antagonist and not the size of the receptor reserve. Such a hypothesis offers much more flexibility in terms of explaining the blockade produced by irreversible agents than does the "spare receptor" hypothesis.

It would be possible, and indeed probable, that in the absence of a scavenger for aziridinium ion such as thiosulphate, the higher concentration of free aziridinium ion in the medium would saturate the "depression" site and thus cause depression of the maximum response before complete alkylation of the "shift" sites. This was found in our studies (Fig. 38) and those of Cook (1971) and can be explained by the binding of phenoxybenzamine to two sites. It must be remembered that although the "shift" site appears to be very readily alkylated, the concentration of the β-haloalkylamine is very important when considering the magnitude of shift obtained.

There is no reason to suppose that all β-haloalkylamines should produce the same degree of allosteric perturbation to the receptor. This could also account for the differing magnitudes of shift of the dose-response curves before the depression of maximum. It is suggestive, however, that the dose-response curves with depressed maxima caused by the three β-haloalkylamines appear to be shifted to the right to the same extent. This implies that the "shift" sites are saturated at this point and that the allosteric perturbation imposed on the receptor by all three agents is the same. At present, there is insufficient data to assume either explanation as being unequivocally correct.

By proposing two binding sites for β-haloalkylamines, the inconsistencies found when explaining the data in terms of spare receptors do not appear. Qualitatively similar is the hypothesis based on the symmetry model proposed by Monod, Wyman, and Changeux for allosteric proteins. This model, however, defines the allosteric component of the blockade in molecular terms and thus warrants serious consideration.

H: MECHANISMS OF ALLOSTERISM IN RECEPTORS

One of the basic tenets of the "two-site" hypothesis is that the alkylation of a site distinct from that required for histamine binding, affects the activation of the receptor by histamine. The mechanism describing this "transduction" of an effect of alkylation on a site by phenoxybenzamine to the histamine binding site can by analogy be given the name allosterism. There are many examples of allosterism to be found when reviewing substrate, inhibitor and activator effects on enzymes (mostly metabolic enzymes except for the protein hemoglobin).

The main reason for considering allosteric interactions is the observed capacity of certain proteins and enzymes to respond to chemical agents possessing structures totally unrelated to that of the substrate molecule. There is a teleological argument for the existence of such a control mechanism when considering enzymic pathways within the cell. Often the rate of a series of enzymic reactions is most easily controlled at a step far-removed from the final formation of the end product of the reaction. The ability of the enzyme requiring the control, to respond to binding of the end product of subsequent reactions (this end product often possessing no structural similarity to the substrate for this enzyme) would thus be an excellent advantage. Such a mechanism would be ideal for controlling protein synthesis in mechanisms such as genetic repression. Monod, Wyman and Changeux (1965) illustrate their conception of allosterism with an appropriate quote from Francis Bacon (1620):

> "It is certain that all bodies whatsoever, though they have no sense, yet they have perception; for when one body is applied to another, there is a kind of election to embrace that which is agreeable, and to exclude or expel that which is ingrate; and whether the body be alterant or altered, evermore a perception precedeth operation; for else all bodies would be like one to another."

Descriptions of how an "allosteric effector" (the agent which affects the binding of the substrate by binding to a distinct site) actually causes the change in the active substrate binding site are, at this point, nebulous. Most often, the effect is described as being due to a conformational change in the protein. One possible mechanism for allosterism in proteins consisting of subunits, would be the movement of the subunits with respect to each other. There are a few instances where allosteric transitions involve the breaking, formation or substitution of bonds between subunits (Monod et al., 1963). Another possibility would be the redistribution of charge of the protein. Although changes in charge distribution usually effect concomitant conformational changes of proteins, it is possible for such a mechanism to produce an undetectable alteration of conformation. It is interesting to note that interaction between the regulatory and active sites of threonine deaminase is lost at high pH. Such behavior implies that that allosteric transitions could depend on ionization of certain critical groups. This is similar to the pH effect on the binding of oxygen to hemoglobin which could be an extension of the allosteric interactions between the subunits of this protein thought to be mediated by a redistribution of charge (for a complete discussion of this topic see Monod _et al_., 1963). There is still insufficient data, however, to determine the exact nature of the allosteric transitions. It is interesting to note, however, that in most enzymic systems shown to exhibit allosterism, this effect is blocked by agents known to react with certain acid groups (i.e., thiol, imidazole). The aziridinium ion, known to react with many nucleophilic groups, would therefore be an excellent candidate as an allosteric effector.

I. THE TWO-STATE MODEL

Another method of explaining the results presented in this thesis is with a two-state model for the histamine receptor. Two-state models have been considered when describing conductance changes observed with agonists on the postsynaptic membrane (for review see Colquhoun, 1975) and the sodium ion-translocating mechanism in epithelia (Cuthbert, 1974a, Cuthbert, 1974b). Although there are a variety of two-state models from which to choose (for a review see Colquhoun, 1973) the one most often utilized is the Monod, Wyman and Changeux (MWC) model also called the symmetry model (1965). The model assumes that the receptor consists of a number of subunits (termed protomers) which form one unit termed the oligomer. It is possible that a receptor be a monomer consisting of only one protomer and in this case the oligomer and the protomer would be one in the same. The protomers would be in one of at least two possible conformations and an axis of symmetry would exist for the oligomer such that the various protomers would occupy equivalent positions. When the ologomer (the receptor) changes conformation from one state to another, its axis of symmetry is conserved; that is all of the protomers would be constrained to adopt the same conformation. Drugs would have different affinities for the two-states of the protomers and would therefore shift the equilibrium between these two-states by binding preferentially to one-state. This would shift the equilibrium between the oligomers (the receptors).

A discussion of how drugs affect this equilibrium between the two receptor forms (termed R, the inactive state and R*, the active state) is given in Chapter I - Section I and need not be repeated. The following discussion will be mainly concerned with the possible mechanisms by which β-haloalkylamines could affect this equilibrium between receptors and subsequently the response to histamine.

Stabilization, by β-haloalkylamine of the inactive form of the receptor would shift the equilibrium further from R^* to R. The result would be an increase of the allosteric constant L ($[R]/[R^*]$). As shown in Appendix I, increases in the allosteric constant can cause parallel shifts of the calculated stimulus-concentration curve with retention of maximum response as defined by the equation

$$\bar{R} = \frac{(1+\alpha)^n}{(1+\alpha)^n + L(1+c\alpha)^n}$$

$\bar{R}$ = fraction of receptors in R^* state

α = normalized ligand concentration ($[\text{agonist}]/K_{aR}$)

n = an "interaction coefficient" - can approach but not exceed the number of identical subunits making up the receptor

L = the "allosteric constant" $[R]/[R^*]$

c = affinity of drug for R/affinity of drug for R^*

It should be stressed that such calculations only serve to show the flexibility of the theoretical approach and cannot be used as valid supportive evidence. It is interesting to note, however, that this approach is capable of predicting many of the experimental findings with β-haloalkylamines.

It has been found that effectors are theoretically capable of changing the Hill plot slope of stimulus-concentration curves like those shown in Fig. 79 (Rubin and Changeux, 1966; Karlin, 1967). Changes in the slope of the Hill plots for dose-response curves to histamine with dibenamine treatment have been observed by Kuhnen-Clausen (1974). A bell shaped curve defines the relation between the maximum slope of the Hill plot and the $\log_{10}$ of the allosteric constant (log L). Thus, not only does this theory predict decreases in the slopes of Hill plots with allosteric effectors but also, depending on the original value of L (in the unblocked receptor system) increases in the slope. The slope of the Hill plot may increase and then decrease as the allosteric constant gets progressively larger. This would be an intriguing possibility when considering the paradoxical increase in the slope of the dose-response curve that is maximally shifted to the right with phenoxybenzamine (Fig. 39). The connection between the slope of the Hill plot and that of the dose-response curve is tenuous however (see Chapter I - Section I and Appendix I) even though the experiment achieving the irreversible shift of the dose-response curve is carried out using the null method (comparison of equiactive doses before and after blockade). A more serious consideration is the quality of data used in these calculations (see Chapter III - Section C).

If β-haloalkylamines produced the shift of the dose-response curve and subsequent depression of the maximum response by progressively increasing the value of the allosteric constant (see Fig 79), then all β-haloalkylamines would predictably produce the same magnitude of parallel shift of the dose-response curve with retention of the maximum response. This would lead to the same incongruities in many of the experimental results presented in Chapter III as did the "spare-receptor" hypothesis. As the results imply that the shift of the dose-response curve and the depression of the maximum response are mediated by separate processes, there must be an alternative mechanism for the depression of the maximum response to occur in this scheme.

It is theoretically possible, however, that the aziridinium ion of phenoxybenzamine depresses the maximum response by directly binding to the R form of the receptor thereby preventing activation to the R* form by histamine. This would be implied by the sensitivity to protection by histamine displayed by the phenoxybenzamine-induced depression of the maximum response. Under these circumstances, the aziridinium ion would increase the value of L as before, but by a different mechanism.

The concentrations of different β-haloalkylamine required to depress the maximum response would depend on the relative affinity of the agent for the two receptor states R and R*. The increases in L (to L') caused by β-haloalkylamine acting as an allosteric effector are given by the following equation (Rubin and Changeux, 1966):

$$L' = L\left[\frac{1 + \beta d}{1 + \beta}\right]^{n}$$

β = concentration of allosteric effector

d = affinity of effector for R/affinity of effector for R^*

Thus it can be seen that increases in β (higher concentrations of phenoxybenzamine) would increase L' and subsequently depress the maximum response. The reverse would be true as well: as a scavenging agent such as BSA or thiosulphate ion removed phenoxybenzamine aziridinium ion from the receptor, L would decrease and the depressed maximum response would return to that of control (as β decreases, L' decreases). Thus the aziridinium ion would be considered to possess "negative efficacy" with respect to the histamine receptor. It would normally be very difficult if not impossible to detect "negative efficacy" for any given drug because the equilibrium, in the absence of agonist, would lie heavily toward the R form of the receptor. As L increases, however, the state function defining $\overline{R}$, the stimulus, becomes much more sensitive to further increases in L thus these could, in theory, be detected. If it is assumed, however, that β-haloalkylamines produce the shift of the dose-response curve and subsequent depression of the maximum response by simply increasing the allosteric constant, albeit at 2 different sites, certain experimental results cannot be explained. In particular, under these circumstances a depression of the maximum response should never occur before the dose-response curve has been shifted 2 log units. All β-haloalkylamines would also produce the same magnitude of shift in the dose-response curve if increases in L were the sole determining factor in the blockade. As seen in Chapter III, both of these effects have been observed thus an alternative must be considered. The most likely possibility would be that β-haloalkylamines modify the differential affinity of histamine for the two receptor forms. If the affinity of histamine is increased for the inactive form or decreased for the active form, at high levels of L, the maximum response would be depressed (see Appendix I and Fig. 81). Such a hypothesis would necessarily imply a second allosteric interaction whereby this change of affinities could be achieved.

This theory would then be a combination of the two-site hypothesis as described by Moran and Triggle (1970) and the two-state hypothesis. Because the differential affinities of the two-sites responsible for the changes in L would not necessarily be the same for all β-haloalkylamines, then different magnitudes in the shifts of the dose-response curves with retention of maximum could occur. The situation would be exactly analogous to that for two binding sites (the "shift" and the "depression" site) only the two-state model provides a molecular mechanism for the shift of the dose-response curve and the depression of the maximum response. Stabilization of the inactive form of the receptor could occur by alkylation of a site distinct from the histamine binding site ("shift" site) through an allosteric mechanism while the depression of the maximum response would be due to reversible binding of the aziridinium ion to the R form of the receptor.

Although this hypothesis is conceptually attractive, there is very little evidence available to test it. It is interesting to note that this hypothesis provides an explanation for the puzzling finding that estimates of affinity for potent agonists are 1/1000 of those of antagonists. If in the absence of drug, the equilibrium between R and R^* lies heavily toward the R form, then the measured affinity constants would be an average of the affinities for the two forms heavily weighted toward R. As the affinity of antagonists for R is by definition much greater than the affinity of agonists for R, dichotomy in the measured affinity constants would be expected. It would also be expected that if the depression of the maximum result was a response of binding of antagonist to the R form, then the facility with which various agents could protect against this blockade would parallel their affinity for the R form (i.e., antagonist > partial agonists > full agonists). This was observed in the protection studies shown in Chapter III - Section T but the chemical interaction between the partial agonist and phenoxybenzamine make the results inconclusive. There is some evidence for the existence of two-states for the cholinergic receptor mediating conductance changes (Rang, 1973; Colquhoun, 1973) although a recent study shows that a range of different conductances can be achieved with various drugs (Colquhoun et al., 1975). Although this result can be interpreted as evidence that the open state of the sodium channel is not the same for all agonists (as predicted by the two-state theory), Colquhoun makes the point that various configurations of the open channel, each having a different conductance, could exist for various drugs. Local effects on pH and field strength also cannot be ignored thus this is not a serious problem in terms of the two-state hypothesis.

None of the studies on the two-state hypothesis reported to date have specifically been concerned with the histamine receptor. Although the transitions between H_1 and an H_2-like receptor occur with temperature in guinea pig ileum (Kenakin et al., 1975; Cook et al., 1975) this only implies that the histamine receptor can exist in more than one state under different physical conditions and has little bearing on the two-state model. There could, however, be a connection between those receptors for which the two-state theory readily applied and the histamine receptor. The two-state hypothesis is well-suited to explain the results with irreversible blockade and also provides a molecular mechanism by which this can occur. Further experimentation is required, however, before conclusive results can be obtained.

J. DISCUSSION OF RELATED TOPICS

The experiments contained in this thesis were designed to elucidate the mechanism of blockade of the histamine receptor by the β-haloalkylamines. Some of these results are interesting in their own right but really have no place in the discussion on spare receptors and allosterism. The three main results in this category are the stimulant properties of the aziridinium ion of DMPEA, the effect of mercaptopyruvate on blockade by phenoxybenzamine and the implications of desensitization on the blockade of the receptor.

i) Stimulant Properties of the DMPEA Aziridinium Ion

The results in Chapter III - Section J indicate that the aziridinium ion of DMPEA is probably responsible for the agonist activity observed in guinea pig ileum. The fact that this agent stimulates muscarinic receptors can be deduced from the differential sensitivity of the muscle to DMPEA-aziridinium ion, acetylcholine and histamine after blockade by SY28, and the pA_2 studies. The one experiment in which the response to the aziridinium ion of DMPEA decreased in a preparation of ileum isolated with an intact nerve supply (after 2 hours of nerve stimulation in the presence of hemicholinium) indicates that DMPEA aziridinium ion may cause release of neuronal acetylcholine. This effect would have to be confirmed. It is interesting to note that the aziridinium ion of another β-haloalkylamine, methyl-2-acetoxyethyl-2'-chloroethylamine (acetylcholine mustard) is also known to stimulate the muscarinic receptors of intestinal smooth muscle (Hirst and Jackson, 1972; Hudgins and Stubbins, 1972; Robinson *et al*., 1975). Although preliminary reports on this agent indicated that it did not irreversibly inactivate the muscarinic receptor (Hirst and Jackson, 1972), it is now known to do so (Hudgins and Stubbins, 1972; Robinson, et al., 1975).

Although DMPEA, an agent known to yield a reactive aziridinium ion capable of alkylation of the adrenergic α-receptor (Kimelberg and Triggle, 1965) and binds well enough to the muscarinic receptor to cause activation, it does not appear to block the response to acetylcholine. The times of exposure of the tissue to the DMPEA, however, were relatively short, thus the full extent of this lack of inhibitory activity is not known. If, however, the aziridinium ion of DMPEA is shown to be completely devoid of antagonistic activity, it could indicate that antagonism of the muscarinic receptor was occurring at a site distinct from the acetylcholine binding site. There would always be the possibility that the binding of the aziridinium ion to the receptor is such that alkylation and therefore inactivation cannot take place. This would be analogous to the depression of the maximum response to histamine by the reversible but not covalent binding of the aziridinium ion of phenoxybenzamine. The difference, however, is that the DMPEA aziridinium ion binds well enough to cause receptor activation while the phenoxybenzamine aziridinium ion does not.

ii) Reversal of Phenoxybenzamine Blockade by Mercaptopyruvate

Although it is tempting to attribute the effects of mercaptopyruvate on the phenoxybenzamine-induced depression of the maximum response (Chapter III - Section H) to interference at an intracellular locus, the results do not support this hypothesis. Since there was no detectable thiosulphate ion present in tissues that were pretreated with mercaptopyruvate, the observed reversal of blockade must be considered a consequence of the ability of the parent compound to react with aziridinium ion. Mercaptopyruvate is capable of reacting with aziridinium ion (Fästh and Sorbo, 1973) presumably at the thiol group.

It would be expected that if phenoxybenzamine was causing the depression of the maximum response to histamine by inactivating some intracellular process, intracellular mercaptopyruvate (by virtue of the fact that it can scavenge aziridinium ion) would selectively prevent or partially protect against the depression of the maximum response to histamine. No such protection was observed, thus while the possibility of a component of the blockade of the histamine response being due to an intracellular alkylation cannot be ruled out by this one series of experiments, it is unlikely.

The observed increase in the selective rate of recovery from the phenoxybenzamine-induced depression of the maximum response is most likely due to mercaptopyruvate functioning as an extracellular scavenging agent much like BSA or thiosulphate ion. It is interesting to speculate on the origin of this mercaptopyruvate since the tissues are washed regularly for many hours. Mercaptopyruvate may either be tightly sequestered at some extracellular locus and be slowly diffusing into the medium (much like the uncyclized phenoxybenzamine under certain circumstances - Chapter III - Section Q) or diffusing into the extracellular space from a pool located within the cell. The results cannot at this point, differentiate between these two possibilities.

iii) Desensitization as Studied with Phenoxybenzamine

As stated in Chapter I - Section K, it is still unclear whether desensitization to histamine is related to the excitation-contraction mechanism or a receptor effect. The action of phenoxybenzamine, at the concentrations utilized in the experiments in this thesis, appears to be confined to the receptor level thus using this agent, it becomes possible to derive information concerning the origins of desensitization.

Irreversible agents possess certain advantages over reversibly acting drugs in the study of desensitization. The antagonism produced by exposure of a highly desensitized preparation, to phenoxybenzamine, can be studied after complete recovery from the complicating effects of the desensitization.

When using these antagonists to determine whether or not desensitization is associated with changes at the receptor level, certain basic criteria must be satisfied.

It must first be established that phenoxybenzamine, at the concentrations and exposure times employed, is binding to the extracellular surface of the receptor macromolecule. Although the uncyclized form of this agent is known to enter the cells of the pancreas (Graham et al., 1968) and vas deferens (Graham et al., 1971), the experimental conditions in these experiments led to much higher concentrations of phenoxybenzamine and longer exposure times that those utilized in this present study. There is much evidence available that suggests that at low concentrations, this antagonist binds to the extracellular surface of the smooth muscle cell membrane. Furthermore, the blockade is largely confined to the histamine receptor as only minimal antagonism of other agonists is observed at the concentrations and exposure times employed (Figs. 36, 37). These results strongly imply that phenoxybenzamine, under these conditions, binds to the H_1 receptor macromolecule. There is no reason to suppose, however, that the irreversible agent binds to the histamine binding site, thus the definition of "receptor phenomenon" can only refer to binding of phenoxybenzamine to some site on the receptor protein either allosterically linked to or, in some other way, intimately associated with the histamine-receptive active site.

If desensitization involves conformational changes in the receptor macromolecule then it might be supposed that these conformational changes would affect phenoxybenzamine binding as well. In this regard, it was found that maximum desensitization produced a statistically significant difference in the ability of phenoxybenzamine to both shift a dose-response curve and depress the maximum response. If, however, these differences are a result of the desensitization, then some correlation should exist between ρ and the measured differences in blockade. No such correlation can be demonstrated for the differences in parallel shift of the dose-response curve thus it is difficult to interpret the significance of the 0.39 log unit difference in the phenoxybenzamine-induced shift of the dose-response curve found when comparing desensitized and non-desensitized tissues. It is possible that desensitization only minimally affects the binding of the phenoxybenzamine responsible for the shift in dose-response curve and therefore differences become apparent in only highly desensitized tissues.

The situation is somewhat less complicated when considering the phenoxybenzamine-induced depression of the maximum response to histamine.

As seen in Fig. 65, there is a correlation between ρ (as a measure of desensitization) and Δmax (the difference between the maximum responses of desensitized and non-desensitized tissues after both are treated with phenoxybenzamine) thus it appears that desensitization involves a change at the receptor level. Due to the non-specific component of desensitization observed (Fig. 63) the magnitude of ρ cannot be considered accurate. Furthermore, it is impossible to assign a unique value of ρ to a tissue blocked with phenoxybenzamine, as the preparation is recovering from the desensitization during the 3 minute exposure to antagonist (ρ changes with time). Thus, the actual percentage values of ρ, shown on the correlation plot, can only be regarded as estimates.

These results suggest that a desensitized receptor does not bind aziridinium ion with quite the facility of a non-desensitized receptor. This finding is similar to those of Lester (1972) in studies with cobra toxin on cholinergic receptors, Miledi and Potter (1971) with α-bungarotoxin on muscarinic receptors, and Dryden and Harvey (1974) with α-bungarotoxin on skeletal muscle cells in culture. Rang and Ritter (1970) found that desensitized cholinergic receptors were more easily antagonized by certain β-haolalkylamines and subsequently termed the phenomenon the "metaphilic effect". As the correlation between ρ and measure of irreversible blockade in these studies is opposite to that observed by Rang (1973), the H_1 receptor may be thought to display a "reverse metaphilic effect" with respect to phenoxybenzamine.

In molecular terms, this effect is best described by the model for desensitization for cholinergic receptors described by Katz and Thesleff (1957). Termed the cyclic model, it defines an equilibrium between normal and desensitized receptors indicated by R and R' respectively.

$$\begin{tikzcd}
A + R \arrow[r, shift left, "fast"] & AR \arrow[l, shift left] \arrow[r, dotted] \arrow[d] & \text{Response} \\
A + R' \arrow[u, "slow"] \arrow[r, shift left] & AR' \arrow[l, shift left, "fast"]
\end{tikzcd}$$

If the aziridinium ion of phenoxybenzamine causes depression of maximum response by binding to the R form of the receptor, then desensitization of a portion of the receptors would essentially remove much of the binding site for the blocking agent. Recovery from desensitization would regenerate active receptor (R' would convert to R) thus there would be a substantial amount of unblocked R and the maximum response in such a tissue would be much less depressed. The data indicate that the alkylation by phenoxybenzamine to cause the parallel shift in dose-response curve is much less dependent on desensitization and it would thus appear that the site which binds phenoxybenzamine to cause the shift in dose-response curve is only minimally altered by the process of desensitization.

The Katz and Thesleff (1957) model of desensitization describes a desensitized receptor which is conformationally different from a normal receptor in the presence (AR') and absence (R') of agonist. It is possible, however, that the histamine molecule remains tightly bound to the receptor for a considerable length of time thereby causing protection against phenoxybenzamine binding. The experiments in which histamine (10^{-6}M) was present during exposure to antagonist and caused little difference in blockade would argue against a long lasting drug-receptor complex as a representation of the desensitized receptor since this concentration of agonist is sufficient to cause maximum response in a non-desensitized tissue. This point of view is valid if a receptor reserve for histamine is not present in this preparation. Even if there is a significant receptor reserve, the possibility of a tightly bound histamine-receptor complex as a representation of desensitization appears unlikely as the presence of 100 times the concentration of agonist causes less difference in the blockade than does desensitization. This concentration of histamine (10^{-4}M) is sufficient to bind to 100% of the receptors assuming a 99% receptor reserve.

A possible mechanism for desensitization to histamine in trachea involving prostaglandin release has recently been suggested (Grodzinska et al., 1975), Exposure of this preparation to high doses of histamine causes release of a prostaglandin E-like substance which could either cause a relaxation of the smooth muscle, as found by Farmer and co-workers (1972), (physiological antagonism) or a specific antagonism of the histamine receptor. Although spontaneous release of prostaglandin has been observed in guinea pig ileum (Davison et al., 1972; Botting and Salzmann, 1974) and although prostaglandins have been proposed to be intimately involved with the histamine response in this preparation (Eckenfels and Vane, 1972), no specific release, as a result of histamine stimulation, has been characterized. It is interesting to note that prostaglandin-like substances could possibly chemically inactivate phenoxybenzamine, as the carboxylic acid moiety of prostaglandins could form an ester with the β-haloalkylamine and thereby reduce the antagonism. At present, the prostaglandins studied in guinea pig ileum have all caused contraction (Bennet et al., 1968; Harry, 1968) and thus could not participate in such a mechanism.

Furthermore, to cause any noticeable effect in phenoxybenzamine blockade, a great deal more prostaglandin-like material would have to be released, than has been reported, for our preparation (~ 0.6 $ng \cdot g^{-1} min^{-1}$, Botting and Salzmann, 1974).

It appears that receptor level binding of phenoxybenzamine is affected by desensitization and that therefore this desensitization involves some kind of perturbation at the H_1 receptor. It is interesting to note that while desensitization profoundly affects the depression of maximum response by phenoxybenzamine, it does not appear to have such effects on the parallel shift induced in the dose-response curve by this agent thereby further implying separate mechanisms for these processes. The concepts discussed here raise interesting questions regarding the high doses of agonist required for receptor protection and the role of desensitization.

V SUMMARY AND CONCLUSIONS

Basically three facts were known when this project began. They were 1) β-haloalkylamines produce an irreversible blockade of the histamine response the prime feature of which is a shift of the dose-response curve with no concomitant depression of the maximum response, 2) the existing theories of drug action (occupation theory i.e., the Michaelis-Menten approach) had no ready explanation for this type of blockade, 3) the most commonly cited explanation for this effect was the "receptor reserve" hypothesis (although there was an alternative in the "two-site" theory put forth by Moran and Triggle, 1970). Superficially the problem appeared to be one of differentiating between two hypotheses but it soon became apparent that it was more a question of basic receptor mechanisms and the consideration of allosterism in these macromolecules.

Since the original proposal in 1956, spare receptors have been shown to be the exception and not the rule. The last two systems for which they appeared valid were the muscarinic receptor of rat jejunum and the histamine receptor system of guinea pig ileum. Through rather elegant experiments, Moran and Triggle (1970) put forth a strong alternative for the muscarinic receptor and certain findings by Cook (1971) cast doubts on the validity for the scheme in guinea pig ileum for histamine. From the experiments discussed in this thesis, it appeared that this paradoxical shift of the dose-response curve was due to a covalently bound species. Many of the results also implied that this β-haloalkylamine-induced shift of the dose-response curve and the depression of the maximum response were the result of separate mechanisms mediated by separate binding sites. This could not be reconciled with the "receptor reserve" hypothesis thus an alternative had to be considered.

The experimental findings were amenable to explanation by a "two-site" theory much like the one proposed by Moran and Triggle (1970). It also fit very well into a relatively new concept in receptor-activation called the "two-state" hypothesis. Both of these theories utilized the concept of allosterism for receptors.

From the experiments discussed in this thesis, it is impossible to differentiate between these two hypotheses. It may not, however, be necessary as the essential points of both can be combined to make a satisfactory alternative. The most plausible form of the "two-state" theory requires two binding sites for the antagonist on the receptor. Since the results of many experiments imply that one site is alkylated by phenoxybenzamine and one binds the aziridinium ion, these should logically be the two species involved at the two respective binding sites.

The mechanism whereby both of these species alter an existing equilibrium between two forms of receptor (defined by L) is particularly attractive as no special assumptions about the receptor system are required to accomodate β-haloalkylamines. It must be stressed, however, that although the "two-state" hypothesis is conceptually ideal, very little other evidence is available on which to consider this theory.

An existing hypothesis for which there are precedents (like the "two-state" hypothesis) and which contains explanations for all experimental findings, is always appealing. Care must be taken, however, in interpreting results from isolated tissue experiments. The Monod, Wyman and Changeux model for allosteric proteins is complex but well defined for isolated proteins and enzymes. When dealing with integral membrane proteins such as receptors, effects on the contractile machinery must be avoided if unequivocal results are to be obtained. Although the concentratons of β-haloalkylamine utilized in these studies appear to affect only the response to histamine, these agents are notorious for their non-specificity. Interpretations of effects such as cooperativity and allosterism should thus be made with caution. Experiments based on the null method which compare effects on the tissue before and after blockade are better than evaluations of response per se.

From these considerations it can be said that the results of β-haloalkylamine blockade of the histamine receptor appear to substantiate the existence of some form of allosterism in the histamine receptor. It also seems that the conventional "receptor reserve" theory proposing the alkylation of a homogenous population of receptors is untenable in light of the inconsistencies encountered between this theory and the experimental results. It would perhaps be more correct to view these antagonists as allosteric effectors and not receptor alkylating agents.

VI FUTURE CONSIDERATIONS

It is of fundamental importance to elucidate possible allosteric mechanisms for receptors. The following are some general considerations for future experiments.

1) If two-states for the receptor exist in an equilibrium, then other agents which function as allosteric effectors in enzyme systems could yield interesting results. Such studies are somewhat constrained in isolated tissue work due to toxicity to the living preparation but sulphydryl reagents and other alkylating agents (i.e., aryldiazonium salts) could prove useful. In particular, alkylating agents which are reported to be specific for imidazole (i.e., iodoacetate) would be interesting to study since certain studies by Rocha e Silva and others imply that the histamine receptor contains a histidine residue at or near the active site. The compound N-ethyl-N(2-chloroethyl)-histamine could also show interesting properties.

2) At present, all attempts to differentiate the predictions of the two-state hypothesis and the Michaelis-Menten approach for receptors have failed (for review see Colquhoun, 1973). Contrasting the behavior of a receptor-equilibrium towards other drugs before and after treatment with an allosteric effector could yield useful results. In particular, the behavior of the tissue toward reversible antagonists could change after treatment with agents which shift the equilibrium between the two receptor forms (see Chapter IV - Section I).

3) It is interesting, at this point, to consider a rather serendipidous finding for DMPEA. Although this agent produced no antagonism of the histamine response in the control curve (10^{-5}M - see Fig. 58)

large depressions of the maximum responses to histamine were encountered with DMPEA after the dose-response curve for the tissue was shifted 2 log units with phenoxybenzamine. Owing to the limited number of experiments, these results have not been included. It is possible to explain this effect by proposing that DMPEA increases the affinity of histamine for the R form of the receptor or decreases it for R^* (i.e., increases c). The control stimulus-concentration curve for a receptor consisting of subunits ($n > 1$) is insensitive to hundred-fold changes in the value of c. Small increases in c at higher values of L (i.e., curve shifted by β-haloalkylamine) cause profound depression of the maximum response.

The peculiar finding that DMPEA produces depression of the maximum response to histamine raises many interesting questions. If this agent were increasing the affinity of histamine for the R form of the receptor (see Chapter IV - Section I), then it might be expected that protection against phenoxybenzamine blockade with histamine would be enhanced after pretreatment with DMPEA. This effect would be an example of how a drug, in this case DMPEA, has little observable effect on the response before but a large effect after treatment with an allosteric effector (i.e., phenoxybenzamine).

4) There is no reason to suppose that a part of the depression of the maximum response to partial agonists is not due to aziridinium ion binding and not alkylation. Under these circumstances it would be possible to reverse with BSA/thiosulphate a portion of the depression of the maximum response up to a point where the limiting factor was the stabilization of the R form of the receptor due to alkylation. Such a partial reversal of blockade would not be consistent with the

"receptor reserve" hypothesis. It would be expected that an agent which had a lower reactivity to the "shift site" and higher affinity for the "depression site" (i.e., ECP) would demonstrate this effect most readily.

5) It has been noted that in tissues which are not as sensitive to histamine as those normally encountered (i.e., ED50 5 x 10^{-7}M instead of 5 x 10^{-8}M histamine) concentrations of phenoxybenzamine which normally caused a 2 log unit of the dose-response curve only produced shifts of 1 log unit. It appeared as though, irrespective of the ED50 for the control dose-response curve, the shifted curve had an ED50 of approximately 5 x 10^{-6}M. It would be expected that the β-haloalkylamine should produce a 2 log unit shift in the dose-response curve under all circumstances. If a portion of the receptors were inoperative, then depression of the maximum response should occur. This was not observed.

If, by some mechanism (i.e., anoxia; trauma) the equilibrium between R and R^* were already shifted to the R form during the determination of the control dose-response curve, then the abnormally high ED50 would be observed. Treatment with phenoxybenzamine, as there is no reason to suppose that the binding sites for the β-haloalkylamine would change the antagonist, would stabilize the R form of the receptor and raise the allosteric constant L to the same value as that for a sensitive tissue. The ED50 for the blocked dose-response curve would be the same irrespective of the ED50 for the control tissue. This effect could be investigated further.

Since the first reports of the sensitivity of guinea pig ileum to various agonists in 1909, almost every agent or combination of agents seems to have been tried in this preparation. Despite this, we are still unclear as to the exact receptor mechanism by which responses are produced. The "receptor reserve" hypothesis contained much appeal since it provided a simple explanation of some unexpected findings and did not require major modifications to existing theories. The much more complicated hypothesis produced here are produced of necessity, rather than desire, in response to observations which are not compatible with the original concept of "spare receptors". It remains to be seen whether the "two-site" or "two-state" models similarly fall to yet another idea, but if these concepts produce the stimulus for further experimental work in molecular pharmacology, they will have more than justified their existance.

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